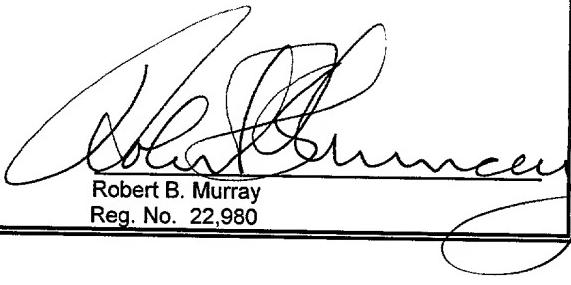


79 Rec'd PCT/PTC 21 SEP 1999

FORM PTO-1390 (REV 5-93)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY DOCKET NO. P564-9039
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		DATE: September 21, 1999
		U.S. APPLN. NO. (IF KNOWN, SEE 37 CFR 1.5) <b>09/381286</b>
INTERNATIONAL APPLICATION NO. PCT/EP98/01653	INTERNATIONAL FILING DATE 20 March 1998	PRIORITY DATE CLAIMED 21 March 1997
TITLE OF INVENTION: PROCESS FOR THE PURIFICATION AND CRYSTALLIZATION OF PROTEASOME		
APPLICANT(S) FOR DO/EO/US: Michael GROLL, Robert HUBER, Lars DITZEL, Richard ENGH		
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. (THE BASIC FILING FEE IS ATTACHED)</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT articles 22 and 39(1).</p> <p>4. <input checked="" type="checkbox"/> A proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)</p> <p>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input checked="" type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input checked="" type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>Items 11. to 16. below concern other document(s) or information included:</p> <p>11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input checked="" type="checkbox"/> Other items or information: PCT/IPEA/416, PCT/IPEA/409, 8/27/99 Letter to WIPO, PCT/RO/101, PCT/IB/306 dated 9/3/99, PCT/IB/306 dated 2/5/99, Small Entity Declaration CHECK NO. 2086 / Drawings - 14 sheets</p>		

U.S. APPLN. NO. (IF KNOWN, SEE 37 C.F.R. 1.50) <b>09/381286</b>		INTERNATIONAL APPLICATION NO. PCT/EP98/01653		ATTORNEY DOCKET NO. P564-9039																				
				DATE: September 21, 1999																				
<p>17. <input checked="" type="checkbox"/> The following fees are submitted:</p> <p><b>Basic National Fee (37 CFR 1.492(a)(1)-(5):</b></p> <table> <tr> <td>Search Report has been prepared by the EPO or JPO.....</td> <td>\$840.00</td> </tr> <tr> <td>International preliminary examination fee paid to USPTO (37 CFR 1.482)...</td> <td>\$670.00</td> </tr> <tr> <td>No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....</td> <td>\$760.00</td> </tr> <tr> <td>Neither international preliminary examination fee (37 CFR 1.482) or international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....</td> <td>\$970.00</td> </tr> <tr> <td>International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) .....</td> <td>\$ 96.00</td> </tr> </table>					Search Report has been prepared by the EPO or JPO.....	\$840.00	International preliminary examination fee paid to USPTO (37 CFR 1.482)...	\$670.00	No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....	\$760.00	Neither international preliminary examination fee (37 CFR 1.482) or international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....	\$970.00	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) .....	\$ 96.00										
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<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b> \$840																								
<p>Surcharge of \$130.00 for furnishing the oath or declaration later than <u>20</u> <u>30</u> months from the earliest claimed priority date (37 CFR 1.492(e)).</p> <table> <tr> <td>Claims</td> <td>Number Filed</td> <td>Number Extra</td> <td>Rate</td> <td></td> </tr> <tr> <td>Total Claims</td> <td>20 - 20 =</td> <td>00</td> <td>X \$ 18.00</td> <td>\$00</td> </tr> <tr> <td>Independent Claims</td> <td>03 - 3 =</td> <td>00</td> <td>X \$ 78.00</td> <td>\$00</td> </tr> <tr> <td colspan="3">Multiple dependent claim(s) (if applicable)</td> <td>+ \$260.00</td> <td>\$00</td> </tr> </table>					Claims	Number Filed	Number Extra	Rate		Total Claims	20 - 20 =	00	X \$ 18.00	\$00	Independent Claims	03 - 3 =	00	X \$ 78.00	\$00	Multiple dependent claim(s) (if applicable)			+ \$260.00	\$00
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<b>TOTAL OF ABOVE CALCULATIONS =</b> \$840																								
<p>Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).</p> <table> <tr> <td>SUBTOTAL =</td> <td>\$420</td> <td></td> </tr> <tr> <td>Processing fee of \$130.00 for furnishing the English translation later the <u>20</u> <u>30</u> months from the earliest claimed priority date (37 CFR 1.492(f)).</td> <td>\$00</td> <td></td> </tr> </table>					SUBTOTAL =	\$420		Processing fee of \$130.00 for furnishing the English translation later the <u>20</u> <u>30</u> months from the earliest claimed priority date (37 CFR 1.492(f)).	\$00															
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<p>Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property</p> <table> <tr> <td>\$00</td> <td></td> </tr> <tr> <td>+ \$40.00</td> <td></td> </tr> </table>					\$00		+ \$40.00																	
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<p>a. <input checked="" type="checkbox"/> A check in the amount of \$420 to cover the above fees is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. <u>14-1060</u> in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>14-1060</u>.</p>																								
<p><b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b></p>																								
<p>SEND ALL CORRESPONDENCE TO:</p> <p>NIKAIKO, MARMELSTEIN, MURRAY AND ORAM LLP Metropolitan Square 655 15th Street, N.W. Suite 330 - G Street Lobby Washington, D.C. 20005-5701 Telephone No. (202) 638-5000</p>																								
 <p>Robert B. Murray Reg. No. 22,980</p>																								

09/381286  
420 Rec'd PCT/PTO 21 SEP 1999

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Michael GROLL et al

Serial No.: Unknown

Filed: September 21, 1999

For: PROCESS FOR THE PURIFICATION AND CRYSTALLIZATION OF PROTEASOME

**PRELIMINARY AMENDMENT**

Assistant Commissioner  
for Patents  
Washington, D.C. 20231

September 21, 1999

Sir:

Prior to calculation of the filing fee and prior to the examination of this application, please amend the above-identified application as follows:

**IN THE CLAIMS:**

Please amend the claims as follows:

Claim 4, line 1, delete "one of the claims 1 to 3" and insert therefor --claim 1--.

Claim 5, line 1, delete "one of the claims 1 to 4" and insert therefor --claim 1--.

Claim 6, lines 2 and 3, delete "one of the claims 1 to 4" and insert therefor  
--claim 1--.

Claim 10, line 1, delete "or 9".

Claim 12, line 1, delete "one of the claims 6 to 11" and insert therefor --claim 6--.

Claim 14, line 1, delete "one of the claims 6 to 13" and insert therefor --claim 6--.

Claim 15, line 2, delete "one of the claims 6 to 14" and insert therefor --claim 6--.

Claim 16, line 3, delete "one of the claims 8 to 14" and insert therefor --claim 8--.

Claim 18, line 1, delete "one of the claims 15 to 17" and insert therefor --claim 15--.

Claim 20, lines 5 and 6, delete "one of the claims 8 to 14" and insert therefor

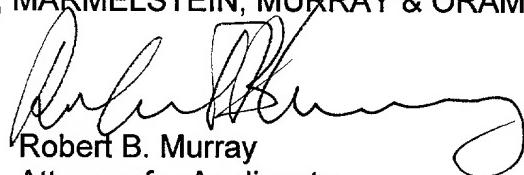
--claim 8--.

REMARKS

The above amendment to the claims has been made to correct the multiple dependency of the claims and to put the application in better condition for examination.

In the event that any fees are due in connection with this paper, please charge our Deposit Account No. 14-1060.

Respectfully submitted,  
NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP



Robert B. Murray  
Attorney for Applicants  
Reg. No. 22,980

Atty. Docket No.: P564-9039

Metropolitan Square  
655 15th Street, N. W.  
Suite 330 - G Street Lobby  
Washington, D. C. 20005-5701  
Tel (202) 638-5000  
Fax (202) 638-4810

RBM/cb

Applicant or Patentee: \_\_\_\_\_

Attorney's  
Docket No.: \_\_\_\_\_

Serial or Patent No.: \_\_\_\_\_

Filed or Issued: \_\_\_\_\_

For: \_\_\_\_\_

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
(37 CFR 1.9(f) and 1.27 (d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.  
ADDRESS OF ORGANIZATION Hofgartenstr. 8, D-80539 München, Germany

TYPE OF ORGANIZATION

- [ ] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION  
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501 (c) (3))  
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA  
(NAME OF STATE \_\_\_\_\_)  
(CITATION OF STATUTE \_\_\_\_\_)  
 WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) AND 501(c) (3) IF LOCATED IN THE UNITED STATES OF AMERICA  
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA  
(NAME OF STATE \_\_\_\_\_)  
(CITATION OF STATUTE \_\_\_\_\_)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled Process for the purification and crystallization of proteasome by inventor(s) Michael Groll, Robert Huber, Lars Ditzel, Richard Engh described in

- [ ] the specification filed herewith  
[ ] application serial no. \_\_\_\_\_, filed \_\_\_\_\_  
[ ] patent no. \_\_\_\_\_, issued \_\_\_\_\_

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below \* and no rights to the invention are held by any person, other than the inventor, who could not qualify as small business concern under 37 CFR 1.9 (d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9 (d) or a nonprofit organization under 37 CFR 1.9(e). \*  
NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities.  
(37 CFR 1.27)

NAME \_\_\_\_\_  
ADDRESS \_\_\_\_\_

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME \_\_\_\_\_  
ADDRESS \_\_\_\_\_

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed. Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.

NAME OF PERSON SIGNING Christa Herzog

TITLE IN ORGANIZATION Head of patent department

ADDRESS OF PERSON SIGNING Hofgartenstr. 8, 80539 München

SIGNATURE Christa Herzog 26.8.99

## PROCESS FOR THE PURIFICATION AND CRYSTALLIZATION OF PROTEASOME

**Description**

The invention concerns a process for isolating a purified eukaryotic crystallizable proteasome preparation and the proteasome preparation obtainable by the process. In addition the invention concerns a purified eukaryotic proteasome preparation in a crystallized form. With the aid of the crystal data from this proteasome preparation it is possible to identify and obtain new proteasome inhibitors, especially with the aid of computer-aided modelling programs.

Proteasome is the central enzyme in protein degradation in the cytosol as well as in the cell nucleus. It is involved in many biological processes including the removal of abnormal, misfolded or falsely assembled proteins, the reaction to stress (by processing or degradation of transcription regulators), cell cycle control (by degradation of cyclines), cell differentiation and metabolic adaptation (by destruction of transcription factors or metabolic enzymes) and the cellular immune reaction (by generation of antigenic peptides that are presented by MHC class I molecules). These cellular functions which are based on a ubiquitin and ATP-dependent degradation of proteins require the 26S proteasome whose nucleus and proteolytic chamber is formed by the 20S proteasome.

The 20S proteasome from *Archaeobacterium Thermoplasma acidophilum* was analysed by X-ray structure crystallography at a resolution of 0.34 nm. It has a

cylindrical shape with a length of 14.8 nm and a maximum and minimum diameter of 11.3 nm and 7.5 nm respectively. It is composed of 28 subunits which are arranged in a particle as 4 homoheptameric rings  $\alpha_7\beta_7\beta_7\alpha_7$  with D7 symmetry (Löwe et al., (1995), Science 268, 533-539). In the *T.acidophilum* proteasome the N-terminal threonine residue of the  $\beta$  subunits is the binding site of inhibitory peptide aldehydes and is essential for the hydrolytic activity. Stock et al. ((1996), Current Opinion in Biotechnology, 7: 376-385) also describe the structure and function of *T.acidophilum* proteasomes. The *T. acidophilum* data cannot be used for eukaryotic proteasomes since the homology of the proteasomes between these species is too low.

Eukaryotic proteasomes are considerably more complex than the archaebacterial proteasome. Thus the 20S proteasome from *Saccharomyces cerevisiae* is composed of a total of seven different  $\alpha$  type and seven different  $\beta$  type subunits which have already been cloned and sequenced cf. e.g. Heinemeyer et al. (1994), Biochemistry 33, 12229-12237).

The eukaryotic 20S proteasomes e.g. from yeast and from mammals are very closely related with regard to the amino acid sequences of subunits and their coarse structure recognizable by electron microscopy. The  $\alpha$  type and  $\beta$  type subunits of the mammalian 20S proteasome form an ordered and well-defined structure (Kopp et al. (1995), J. Mol. Biol. 248, 264-272). In mammalian cells three additional non-essential subunits of the 20S proteasome which are named LMP2, LMP7 and MECL1 can replace constitutive components after induction with the cytokine interferon  $\gamma$ . Their expression or specific deletion changes the peptidase specificity of the

proteasome and the expression rate of MHC class I molecules on the cell surface.

Hilt, Heinemeyer and Wolf ((1993), Enzyme Protein 47: 189-201) describe the structure of 20S and 26S yeast proteasomes and the proteolytic activity of  $\beta$  type subunits. In addition the different functions of 20S and 26S proteasomes are discussed with regard to the metabolism and differentiation of a cell. Crystallographic data of eukaryotic proteasomes are not described.

Starting materials that have previously been used to purify proteasomes such as tissue and cells of mammals such as mouse, rat, human or bovine, other animals, plants and yeast are listed in the publication by Rivett et al. (1994), Methods Enzymol. 244, 331-350) and the citations included therein.

There are also numerous documents in the patent literature which relate to proteasomes. Thus for example the production of eukaryotic proteasomes is described in EP-A-03 45 750, JP-A-05 292 964 and JP-A-06 022 759. However, the proteasome preparations that are disclosed do not have sufficient purity to enable crystallization.

Morimoto et al. ((1995), J. Biochem. 117, 471-474), Hwang et al. ((1994), Mol. Cells, vol. 4, 273-275) and Perkins et al. ((1994), Journal of Structural Biology 113, 124-134) describe the crystallization of eukaryotic proteasomes. Resolutions of only 0.44 nm, > ca. 5.0 nm and 1.5 nm respectively are achieved due to the low purity of the proteasome preparations so that a structural determination or molecular modelling is not possible with these proteasome preparations.

Nucleotide and amino acid sequences of proteasome subunits are described for example in the Japanese applications JP-A-04 077 497, JP-A-04 077 498, JP-A-04 117 283, JP-A-05 317 059, JP-A-07 255 476, JP-A-08 116 972, JP-A-08 205 871 and JP-A-08 217 796 and in the Japanese Patent 40 51 896.

Proteasome inhibitors are described for example in JP-A-05 000 968, WO 92/20 804, WO 94/17 816, WO 95/24 914, WO 95/25533, WO 96/13 266, WO 96/32 105 (lactacystin analogues) and US-A-55 80 854 (peptide aldehyde inhibitors).

Klafky et al. ((1995), Neuroscience Letters 201, 29-32) examine the effect of the proteasome inhibitor calpain inhibitor 1 on the secretion of  $\beta$  amyloid peptide which is formed by cleavage of the  $\beta$  amyloid precursor protein (APP) and which has been discussed to be a trigger of Alzheimer's disease. There are no proteasome structural data in this publication.

Fenteany et al. ((1995), Science, vol. 268, 726-731) describe the streptomyces metabolite lactacystin as a cell cycle inhibitor and inducer of neurite outgrowth of a mouse neuroblastoma cell line. The 20S proteasome was identified by means of tritium-labelled lactacystin as the specific cellular target of this inhibitor. A crystallizable proteasome preparation is not described.

WO 91/13904 describes the identification and characterization of a chymotrypsin-like protease which is present as a multicatalytic protease and its use for treating Alzheimer's disease. The use of substrates that are specific for chymotrypsin activity to test or screen

for inhibitors as described in this document only leads to the identification of inhibitors that are specific for a chymotrypsin-like activity.

Hence it is apparent that there is a great need for further information about proteasomes especially with regard to their exact structure in order to enable the preparation of new proteasome inhibitors in a rational manner. Thus the object of the invention was to provide a process which enables the crystallization of eukaryotic proteasome preparations so that the crystal structure can be used to simplify the development of new inhibitors.

The inventive object is achieved by a process for isolating a purified eukaryotic proteasome preparation comprising the steps:

- (a) production of a crude extract by lysing eukaryotic cells,
- (b) separation of insoluble components from the crude extract,
- (c) chromatographic separation into fractions by means of an ion exchange medium, e.g. Q-Sepharose,
- (d) testing the fractions obtained in step (c) and collecting the active fractions,
- (e) chromatographic separation over hydroxyapatite,
- (f) testing the fractions obtained in step (e) and collecting the active fractions,
- (g) concentrating the pooled fractions,
- (h) chromatographic separation over a gel filtration medium in a molecular weight range of 5 kD to 5 MD, e.g. Superose and
- (i) testing the fractions obtained in step (h) and collecting the active fractions.

Any eukaryotic cells can be used as a starting material for the process according to the invention e.g. animal cells, plant cells or fungal cells such as yeast cells. The use of yeast cells e.g. *Saccharomyces cerevisiae* is particularly preferred.

The fractions are usually tested during the purification process by determining the typical proteolytic activity for proteasomes. In this connection known chromogenic peptides can for example be used as substrates. The fractions are preferably tested by carrying out two parallel determinations of the proteolytic activity in each case, one of which is carried out in the absence and the other in the presence of a proteasome inhibitor e.g. lactacystin. This type of testing allows the fractions containing proteasomes to be unequivocally differentiated from other fractions with proteolytic activity.

The concentration process comprises three chromatographic separation steps (c), (e) and (h) of which at least one can be carried out in a FPLC system e.g. step (h).

A purified proteasome preparation is obtained by the process according to the invention which is present in an adequate amount and purity to enable a subsequent crystallization.

Hence a further subject matter of the present invention is a purified eukaryotic proteasome preparation which is obtainable by the process according to the invention. Yet a further subject matter of the present invention is a purified eukaryotic proteasome preparation in a

crystallizable form. Yet a further subject matter of the present invention is a purified crystallized eukaryotic proteasome preparation.

The crystallized proteasome preparation can also contain a proteasome inhibitor. Examples of suitable known proteasome inhibitors are lactacystin or analogues thereof and tripeptide aldehydes such as calpain inhibitor.

The eukaryotic proteasome preparation according to the invention comprises a 20S proteasome i.e. a complex of 28 subunits each of which contains two molecules of seven different  $\alpha$  type subunits and seven different  $\beta$  type subunits. In addition the complex can also contain metal ions e.g. magnesium, solvent molecules e.g. water and other polypeptide components.

The purified eukaryotic proteasome preparation according to the invention can be used to identify and isolate new proteasome inhibitors. Data from the crystal structure of crystallized eukaryotic proteasome preparations are used in particular for this. The identification and isolation of new proteasome inhibitors is preferably carried out using a computer-aided modelling program.

For example the inhibitor can be designed by visually inspecting graphic representations of the structure and in particular by

- (a) determining the accessible volumes for ligands at active sites e.g. with the aid of the programs INSIGHT, SYBYL, QUANTA, FRODO, O etc.,
- (b) determining ideal ligand properties with regard to

hydrophobicity or hydrogen bonds e.g. with the aid of the programs LUDI, GRID, etc. or/and

- (c) determining the electronic properties of surfaces that are accessible to ligands at the active sites e.g. with the aid of the program GRASP etc.

Alternatively or additionally it is also possible to determine ligands by automated ligand fragment docking or adaptation procedures eg. with the aid of the programs DOCK, LUDI, LEAPFROG etc.

For this purpose it is particularly preferable to use the crystal data for the proteasome subunits of the  $\beta$  type in particular for the proteasome subunits  $\beta_5/PRE2$ ,  $\beta_1/PRE3$  or/and  $\beta_2/PUP1$  or homologous subunits from other eukaryotic proteasomes and neighbouring subunits thereof e.g.  $\beta_4/C11$  or/and  $\beta_7/PRE4$ .

In order to design inhibitors of the human proteasome it is possible to modify the inventive crystal structural data of the yeast proteasome by homology modelling using known amino acid sequences of the human proteasome. Such a homology modelling can be carried out by molecular graphic programs such as O, INSIGHT, FRODO, etc.. In particular the present invention encompasses a homology modelling of the homologous active sites of the active monomers in general and in particular for the purpose of inhibitor design. The homology of the amino acid sequences of the yeast proteasome and of the human proteasome in the relevant regions is shown in Figure 1.

In addition it is intended to illustrate the invention by the following examples and figures.

Figure 1 shows the homology between the amino acid sequences from yeast and humans coding for the active subunits of the proteasome; the  $\beta_1$ /PRE3,  $\beta_2$ /PUP1,  $\beta_5$ /PRE2 subfamilies are indicated by the yellow, green and blue colour respectively; the residues of the S1 pocket which influence the specificity changes of the PRE3 subfamily after substitution of the human subunit Y by LMP2 after cytokine induction are shown in brown;

Figure 2 shows the topology of the 28 subunits of the 20S proteasome drawn as spheres,

Figure 3 shows the  $C^\alpha$  chain positions of the subunits  $\beta_7$ /PRE4,  $\beta_6$ /C5,  $\beta_1'$ /PRE3,  $\beta_2'$ /PUP1 and  $\beta_3'$ /PUP3 in which the  $\beta$ -cis and  $\beta$ -trans- $\beta$  interactions by contacts of insertion segments are highlighted,

Figure 4a to b show electron density maps (contoured starting at  $1\sigma$ ) in similar orientations around THR1 with two  $F_o$ - $F_c$  coefficients after double averaging; the red parts of the model were omitted via the phasing.  
 $\beta_5$ /PRE2 with the covalently-bound lactacystin (LACT) and the water molecule NUK (a) and  $\beta_7$ /Pre4 with a part of a propeptide (b),

Figure 5 shows a scheme of the proposed chemical steps of autolytic and substrate hydrolysis. Generation of a processing intermediate by hydrolysis on the acidic  $\beta$  ring surface (A).

Generation of the completely processed active subunit via an acyl enzyme (B) and its hydrolysis (C). Michaelis complex of a substrate polypeptide (D). Cleavage on the  $\beta$  ring surface and formation of the acyl enzyme (E) associated with peptide cleavage. Acyl enzyme hydrolysis and release of the octapeptide product (F).

Figure 6a to c show the binding of the calpain inhibitor and the S1 pockets,  $\beta_1/\text{PRE}3$  is shown in grey with the P1 contacting residues shown in red;  $\beta_2/\text{PUP}1$  is shown in green and the inhibitor is shown in blue (a);  $\beta_2/\text{PUP}1$  (b),  $\beta_5/\text{PRE}2$  (c) have an analogous colour scheme;

Figure 7 shows the lower half of the  $\beta$ - $\beta$  chamber. The main chain with the red circles for the carbonyl oxygens is shown for the C-terminal sections of the helices H2 of the seven  $\beta$  type subunits which define the  $\beta$  ring area. The intermediary processed and the unprocessed propeptides of the subunits  $\beta_6/\text{C}5$ ,  $\beta_7/\text{PRE}4$ ,  $\beta_3/\text{PUP}1$  and  $\beta_4/\text{C}11$  (green) and the calpain inhibitor (yellow) bound to  $\beta_1/\text{PRE}3$ ,  $\beta_2/\text{PUP}1$  and  $\beta_5/\text{PRE}2$  are shown. Two magnesium ions that are located near to the  $\beta$  ring area are shown as silver circles; and

Figure 8 shows a surface view of the proteasome molecule cut along the cylinder axis. Three of the six calpain inhibitor molecules bound to  $\beta_1/\text{PRE}3$ ,  $\beta_2/\text{PUP}1$  and  $\beta_5/\text{PRE}2$  are shown in red as space

filling models. The sealed  $\alpha$  openings at the two ends of the particle, a few narrow side windows and the sharply cut inner  $\beta$  ring surfaces can be seen.

### Examples

#### Example 1 Protein preparation and characterization

Yeast cells of *Saccharomyces cerevisiae* (Hefe-Mayr, Munich, Germany) were washed twice with ice-cold water and suspended in buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM NaN<sub>3</sub>) in a weight ratio of cells to buffer of 2:3. The cells were disintegrated for 10 min in a grinder (Biomatik, Germany) with glass balls (diameter: 0.5 mm; volume ratio of glass balls to cell suspension: 3:2). The disruption of the cells was monitored microscopically.

After filtration the crude extract was centrifuged for 10 min at 10,000  $\times$  g in a Sorvall RC 2B centrifuge. The supernatant was again centrifuged for 45 min at 134,000  $\times$  g in a Ti-55.2 rotor (Beckmann). The lipids from the upper most layer were carefully removed and the remaining yellow solutions were combined. The protein concentrations were about 50 mg/ml.

Immediately after centrifugation the extract was applied to a Q-Sepharose column (5 x 20 cm) which had been equilibrated with 280 mM NaCl in buffer A. The column was washed with 280 mM NaCl in buffer A, the proteins were eluted with a gradient of 280 to 800 mM NaCl. The flow rate was 120 ml/h and 12 ml fractions were collected. The proteasome was eluted at 400 - 450 mM

NaCl. Chymotrypsin-like (CL), peptidylglutamyl-peptide-hydrolase (PGPH) and trypsin-like (TL) enzymatic activities were measured in all fractions.

In order to obtain the 20S proteasome the CL activity was again measured in all active fractions in the presence of lactacystin and the fractions with a reduced activity were collected. The combined fractions were diluted three-fold with water and applied to a hydroxyapatite column (3 x 10 cm) which had been equilibrated with 60 mM potassium phosphate, pH 7.5. The column was washed with 60 mM potassium phosphate pH 7.5 and eluted with a gradient of 60-300 mM potassium phosphate. The flow rate was 60 ml/h. 12 ml fractions were collected. The CL, PGPH and TL activity was measured in all fractions and the active fractions were combined.

The combined fractions were concentrated 20-fold by ultrafiltration using a AMICON YM30 membrane and the concentrate was applied to a Superose 6 column (1 x 30 cm) equilibrated with buffer A. The elution was carried out with a flow rate of 18 ml/h in buffer A. The proteasome eluted after 37 min. In this manner it was possible to obtain 50 mg crystallizable protein from 500 g yeast cells.

All preparative steps with the exception of FPLC were carried out at 4°C. The chromogenic peptide substrates were dissolved in dimethyl sulphoxide at a concentration of 1 mM. The proteolytic activity towards these substrates was determined according to Achtstetter et al. (1994), J. Biol. Chem. 259, 13344-13348. The chromogenic peptide substrates were obtained from Bachem

(Bubendorf, Switzerland). Q-Sepharose and hydroxyapatite were obtained from Sigma and BioRad. The FPLC device, the MonoQ and Superose 6 column were obtained from Pharmacia (Freiburg, Germany), all other chemicals were obtained in the highest possible purity from Merck (Darmstadt, Germany).

Example 2 Crystallization

The crystals were grown in hanging drops at 24°C. The protein concentration which was used for crystallization was 40 mg/ml in 10 mM Tris/HCl (pH 7.5) and 1 mM EDTA. The drops were composed of 4 µl of the protein solution and 2 µl of a reservoir solution which contained 40 mM magnesium acetate, 0.1 M morpholinoethane sulfonic acid (pH 6.5) and 12 % 2,4-methylpentanediol. The crystals containing the inhibitor lactacystin were produced by immersion in a 1 mM lactacystin solution for 6 h. The crystals containing the inhibitor acetyl-Leu-Leu-norleucine (calpain inhibitor I, Boehringer Mannheim) were produced by immersion in a 5 mM calpain solution for 6 h. The crystallographic data are shown in Table 1.

Example 3 Crystallography

The crystals were very-well ordered and exhibited only a slight anisotropy. Thus a resolution of 0.24 nm was possible. The acetyl-Leu-Leu-norleucinal-inhibited crystals were slightly less ordered.

The anisotropy of the diffraction was corrected using the found structure factor amplitudes with those that were calculated from a model with isotropic temperature factors using XPLOR (Bruenger, 1992). The data sets were

obtained with the BW6 beam line at the DESY Hamburg using a synchrotron radiation of  $\lambda = 0.11$  nm. The crystals were immersed in an antifreeze buffer (30 % MPD; 28 mM magnesium acetate, 0.1 M morpholinoethane sulfonic acid, pH 6.9) and frozen in a stream of 90°K cold nitrogen gas. The diffraction data were collected with a 300 mm Mar research imaging plate at a distance of 275 mm (LACT) or 280 mm (CAL). The X-ray intensities were determined using the MOSFLM computer program version 5.3 and the data reduction was carried out with CCP4 (Leslie (1992), Acta Cryst. D50, 760-763; Joint CCP4 and ESF-EACMB, Newslett. Protein Crystallogr. (Daresbury Laboratory Warrington UK 26M Collaborative Computational Project Number 4 (1994)).

A rotation function calculated at 0.5 nm resolution showed two peaks that were related to the crystal symmetry which indicates the presence of local diadic molecule axes at  $\psi 86^\circ \phi 90^\circ$  and  $\psi 94^\circ \phi 90^\circ$ . Their correlation values were half the value of the crystallographic diad that would have been expected for an almost ideal molecular two-fold symmetry. The T. acidophilum model was used for the Patterson search calculation using AMoRe (Navaza (1994), Acta Cryst. A50, 157-163) at a resolution of 0.35 nm. This showed that if one takes into consideration the D7 symmetry of the experimental model, there is a single solution with a correlation value of 0.32 and an R factor of 56 % compared to the next-highest peak of 0.28 and 57 %.

The T. acidophilum model was reduced to polyalanine with only a few conserved residues which remained in the  $\alpha$  type subunit. This model yielded an R factor of 57.7 % and was used to calculate a  $2F_o - F_c$  map at 0.24 nm with X-PLOR (Bruenger (1992), X-PLOR version 3.1). A system

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for X-ray crystallography and NMR was used. The electronic density was averaged in real space using MAIN (Turk (1992), Dissertation, Technical University Munich) and using the local double axis in the present model ( $\psi=85.1$ ,  $\phi=90.8$ ,  $\kappa=180.1$ ), transformed back, and a new density was calculated with  $2F_o-F_c$  coefficients. After 10 adjusting cycles the quality of the map was good ( $R_{back}=27.3\%$ ). The individual subunits were identified on the basis of their characteristic insertions, deletions and amino acid sequences and were incorporated into the map on an ESV-30 graphic system work station (Evans & Sutherland, Salt Lake City, Utah) using FRODO (Jones (1978), J. Appl. Cryst. 11, 268-272). A crystallographic refinement was carried out with X-PLOR (Bruenger, 1992) with energetic and double non-crystallographic symmetry constraints using the parameters described by Engh and Huber (1991), Acta Cryst. A 47, 392 - 400. In addition a scattering component for the solvent was calculated in order to correct for an anisotropic crystal arrangement and was incorporated into the calculation of the model during the refinement.

The final model has taken into account the inhibitor molecules lactacystin and acetyl-Leu-Leu-norleucinal, 18 magnesium ions and 1,800 water molecules. The R values are satisfactory and the standard geometry of the bonds and angles are excellent. The local molecular diadic symmetry is well-conserved which is also shown by the very low value  $R_{back}$  13 % in the final step of the analysis. The increase in the R value by 3 % for data with a resolution of 0.28 nm compared to 0.24 nm is a result of the anisotropic crystal arrangement which impairs the data quality and of the limited incorporation of ordered solvent molecules.

Example 4 Characterization of the structure

4.1 Structure of subunits

The 14 genes cloned from yeast which code for components of the 20S proteasome can be divided into seven  $\alpha$  type and seven  $\beta$  type subunits.

The  $\beta$  type subunits are synthesized as precursors which are processed into the mature forms present in the assembled proteasome. The mature  $\beta$  type polypeptides  $\beta_2/PUP1$ ,  $\beta_5/PRE2$  and  $\beta_1/PRE3$  are obtained from their proforms by cleavage between Gly-1 and Thr1 with release of the active site Thr1, whereas  $\beta_7/PRE4$  is cleaved between Asn-9 and Thr-8 and  $\beta_6/C5$  is cleaved between His-10 and Gln-9 and are present as stable processing intermediates.  $\beta_4/C11$  and  $\beta_3/PUP3$  are not processed and begin with Met(-1) or Met(-9) respectively. The subunits PUP1, PRE2 and PRE3 are referred to as completely processed, the subunits PRE4 and C5 as partially processed and the subunits C11 and PUP3 as unprocessed.

All 14 subunits are present in the crystalline molecular structure at well-defined positions. They are almost completely defined by the electron density apart from a few chain termini and long insertion segments.

The electron density for the main chains is defined as follows in the  $\alpha$  type subunits:  $\alpha_2/Y7$ : Thr5-Leu236,  $\alpha_3/Y13$ : Gly4-Gly237,  $\alpha_4/PRE6$ : Tyr8-Gln244,  $\alpha_5/PUP2$ : Arg10- Glu243 (7 residues of the insertion are not defined - Gly12 to Arg 126),  $\alpha_6/PRE5$ : Phe4 - Ile233,  $\alpha_7/C1$ : Gly5 - Asn241,  $\alpha_1/C7$ : Gly6 - Asp240.

In the  $\beta$  type subunits the electron density is defined

as follows:  $\beta_3/\text{PUP3}$ : Ser-8-Asp 193,  $\beta_6/\text{C5}$ : Gln-9-Asp 193,  $\beta_4/\text{C11}$ : Met-1-Gln192,  $\beta_7/\text{PRE4}$ : Thr-8-Ile211,  $\beta_2/\text{PUP1}$ : Thr1-Cys221,  $\beta_1/\text{PRE3}$ : Thr1-Leu196,  $\beta_5/\text{PRE2}$ : Thr1-Gly211.

All seven  $\alpha$  and  $\beta$  type polypeptides have a characteristic  $\beta$  sandwich structure. It is composed of two five-strand antiparallel  $\beta$ -folded sheet structures with the overlying helical layers composed of the helices H3, H4, H5 and the underlying helices H1 and H2. However, they differ in the bends which vary in length by one or two amino acid residues, in long insertions which connect secondary structural elements and in the N-terminal and in particular in the C-terminal regions.

In the  $\alpha$  type subunits  $\alpha_2/\text{Y7}$  has a long insertion loop between the strands S9 and S10 which is composed of a short  $\alpha$  helix and a  $\beta$  strand.  $\alpha_1/\text{C7}$  has an extension of the helix H3 by two bends as a result of the insertion at G180. The subunits  $\alpha_1/\text{C7}$ ,  $\alpha_3/\text{Y13}$ ,  $\alpha_4/\text{PRE6}$ ,  $\alpha_5/\text{PUP2}$  and  $\alpha_7/\text{C1}$  have longer C-terminal helices H5 which protrude into the solution from the particle surface. The highly charged, mostly acidic C-terminal segments are unstructured.

In the case of the  $\beta$  type subunits with long insertions,  $\beta_7/\text{PRE4}$  has a sharp bend between the helices H1 and H2 and an additional  $\alpha$  helix with 2 bends at residue 145.  $\beta_6/\text{C5}$  has an insertion of 17 residues between H3 and H4 with a complex folding and a short helix.  $\beta_2/\text{PUP1}$  has a very long C-terminal extension whose last 11 residues are very disordered. The subunits  $\beta_3/\text{PUP3}$  and  $\beta_6/\text{C5}$  have short C-termini so that the helices H5 do not exist and the strands S10 are extended to enlarge the  $\beta$ -folded

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sheet. The helix H5 exists in  $\beta$ 4/C11 but it is two folds shorter than in the case of *T. acidophilum*.

Many of these subunit-specific folds, insertions and N- and C-termini are involved in the contacts between subunits as discussed in the following.

4.2 The (C7, Y7, Y13, PRE6, PUP2, PRE5, C1; PRE3, PUP1, PUP3, C11, PRE2, C5, PRE4)<sub>2</sub> complex

Each of the seven  $\alpha$  type subunits has two neighbours within the heptameric ring which exhibit  $\alpha$ -cis interactions and one or two neighbouring  $\beta$  type subunits in another ring with  $\alpha$ -trans- $\beta$  interactions. In addition to the  $\beta$ -cis and  $\beta$ -trans- $\alpha$  interactions, the central  $\beta$  type subunits have one or two neighbouring  $\beta$  type subunits in the other  $\beta$  ring with  $\beta$ -trans- $\beta$  interactions.

The general architecture of the quarternary structure is the same in the proteasome of *T. acidophilum* and yeast (Fig. 2): The N-terminal loop segment, helix H0 (residues 20 to 30), loop L, the loop connecting H2 and S5 and the strand S7 mediate  $\alpha$ -cis interactions. The  $\beta$ -cis contacts which appear to be less close include the loop L, the N-end of the helix H1, the strand S7 and the bend linking the strand S8 and the helix H3. These contacts are derived from the D7 symmetrical precursor and are also found in the *T. acidophilum* proteasome. Despite the conserved architecture these contacts are specific for the respective subunits due to their specific amino acid sequences.

Their are many additional contacts which are absent in

T. acidophilum and result from sequences and sequence insertions which are generally characteristic for yeast and eukaryotes. Within the  $\alpha$  rings, close  $\alpha$ -cis contacts are made by the intertwined N-termini of the subunits  $\alpha_1/C7$ ,  $\alpha_2/Y7$ ,  $\alpha_3/Y13$  and  $\alpha_7/C1$  in the centre of the heptameric ring. Tyr8 which is conserved in all subunits plays a central role. Within the  $\beta$  rings there is a very specific contact between  $\beta_2/PUP1$  and  $\beta_3/PUP3$  which is mediated by the long C-terminal arm of PUP1 which includes PUP3 and almost touches the next but one neighbour  $\beta_4/C11$ .  $\beta$ -trans- $\alpha$  contacts are made by the helix H1 loop helix H2 motifs which interact with the same motifs of two neighbouring  $\alpha$  subunits. This basic contact motif was also seen in the T.acidophilum structure (see Figure 4a by Löwe et al. (1995), Science 268, 3479-3486) but the insertion at residue 66 of  $\beta_7/PRE4$  favours its association with  $\alpha_6/PRE5$  and  $\alpha_7/C1$ . In the same manner the long insertion in  $\alpha_2/Y7$  at residue 210 between strands S9 and S10 binds to  $\beta_2/PUP1$  and couples this pair. Specific  $\beta$ -trans- $\beta$  interactions are formed by the C-terminal arm of  $\beta_7/PRE4$  which is intercalated between  $\beta_2'/PUP1$  and  $\beta_1'/PRE3$ . The C-terminal segment of  $\beta_5/PRE2$  interacts with  $\beta_3'/PUP3$  and  $\beta_4'/C11$  in a similar manner (Figure 3). The long insertion of  $\beta_6/C5$  at residue 145 contacts subunit  $\beta_3'/PUP3$  and the C-terminal arm of  $\beta_2'/PUP1$ .

Very specific  $\beta$ -trans- $\beta$  interactions are mediated by magnesium ions: magnesium Y8 bridges the main chain carboxylate of Asp193 from  $\beta_6/C5$  with the loop 162 to 167 of  $\beta_2'/PUP1$ . In the same manner the magnesium Y9 bridges the subunits  $\beta_3/PUP3$  via Asp193 with  $\beta_5'/PRE3$ . In addition these carboxylate groups are ligands for other magnesium ions which are located in the loops 165 of  $\beta_4/PUP3$  (magnesium W6) and  $\beta_6/C5$  (magnesium W4) and

can play a role in stabilizing the subunit structure. The aspartate residues are completely covered and their side chains participate in charge-charge interactions with Arg 19 of  $\beta 2'$ /PUP1 and Arg 19 of  $\beta 5'$ /PRE2 which further strengthens the  $\beta$ -trans- $\beta$  contacts. The  $\beta$  type subunits  $\beta 1$ /PRE3 and  $\beta 4$ /C11 are located at the single molecule diads of the yeast proteasome and are very similar to the dominant  $\beta$ -trans- $\beta$  contact the residues 133-137 of the helix H3 of *T. acidophilum*.

18 magnesium positions were identified in the proteasome molecule of which 12 are located on the inner walls of the  $\beta$ - $\beta$  chamber and which prove the acidic nature of this compartment which is discussed in the following. It can be seen that the numerous specific interactions between the subunits determine their specific and unequivocal positions within the proteasome.

#### 4.3 The N-terminal threonine position

A catalytic system with Thr1, Glu17 and Lys33 was defined in the *T.acidophilum* proteasome by structural and mutation investigations (Löwe et al. (1995), supra and Seemüller et al. (1995), Science 268, 579-582).

The residues Ser129, Ser169 and Asp166 are located close to Thr1 which are required for the structural integrity of this position but could also be involved in the catalysis. It was shown by mutagenesis that Asp166 in the proteasome of *T.acidophilum* is essential (Seemüller et al. (1996), Nature 382, 468-470). These residues are invariant in the active subunits PUP1, PRE2 and PRE3.

In addition a completely bound solvent molecule NUK was

found in all three subunits close to Thr $10^{\gamma}$  and N, Ser $1290^{\gamma}$  and N and Gly $47N$  as shown as an example for the subunit  $\beta 5/PRE2$  in the lactacystin complex (Figure 4). This was not recognized in a lower resolution in the model of *T.acidophilum*. Thr $1N$  has hydrogen bridges to Ser $1680$  and O $^{\gamma}$  and Ser $1290^{\gamma}$ . Thr $10^{\gamma}$  has a hydrogen bridge to Lys $33^{\delta}$ . Asp $17$  has hydrogen bridges via O $^{\delta 1}$  to Arg $19N$  and Gly $170N$  and via O $^{\delta 2}$  to Thr/Ser $2N$  and Lys $33N^{\delta}$ . In a similar manner Lys $33N^{\delta}$  has three hydrogen bridges to Asp $170^{\delta 2}$ , Arg $190$  and Thr $10^{\gamma}$ .

The pattern of hydrogen bridges leads one to assume that Asp $17$  as well as Lys $33$  are charged. Thr $1N$  can form a hydrogen bridge to ThrO $^{\gamma}$  and is presumably neutral, a state which is favoured by a nearby positively charged lysine residue. Such a charge distribution would also be expected from the respective standard pKa values. Thr $1N$  is therefore very probably the proton acceptor, if Thr $10^{\gamma}$  is part of an electrophilic centre. This is confirmed by the structure of the lactacystin complex which has an ester between lactacystin and Thr $1$  as a result of a  $\beta$  lactone ring opening after a nucleophilic attack by Thr $10^{\gamma}$ . Thr $1N$  is at exactly the position to serve as a proton shuttle from Thr $10^{\gamma}$  to lactacystin-06'. An analogous reaction sequence is proposed for the hydrolysis of the C-terminal fluorophores of fluorogenic substrates where the proton transfer takes place in the amide nitrogen of the leaving group. The generated acyl enzyme is deacylated by the water NUK as shown in the sections D-E of figure 5. Alternatively or concurrently NUK could directly attack the peptide bond thus circumventing the intermediate I.

#### 4.4 Inhibitor binding

$\beta_3/PUP1$ ,  $\beta_1/PRE3$  and  $\beta_5/PRE2$  have the inhibitor acetyl-Leu-Leu-norleucinal bound covalently to Thr $10^{\gamma}$  presumably as a hemiacetal. It assumes a  $\beta$  conformation and fills the gap between strands which contain the residues 20 and 21 and 47 (allocated to the loop L in figure 3 by Löwe et al., 1995, *supra*) to which it is bound via hydrogen bridges which generates an antiparallel  $\beta$  folded sheet structure. The norleucine side chain extends into a pocket (the S1 pocket) the side of which is open towards a tunnel which leads to the particle surface. The leucine side chain in P2 is not in contact with protein and the leucine side chain in P3 is in contact with the neighbouring  $\beta$ -subunits. The S1 specificity pocket is mainly formed by the residues 20, 31, 35, 49, 53 i.e. Ala20, Val31, Ile35, Met45, Ala49, Gln53(K) in  $\beta_5/PRE2$  (fig. 6c), Thr20, Thr31, Thr35, Arg45, Ala49, Gln53 in  $\beta_1/PRE3$  (fig. 6a), Ser20, Cys31, His35, Gly45, Ala49, Glu53 in  $\beta_2/PUP1$  (fig. 6b). The residue 45 forms the bottom of the pocket and appears to largely determine its character. Neighbouring subunits in the  $\beta$  rings also contribute to the S1 pockets and modulate their character:  $\beta_2/PUP1$  in the case of  $\beta_1/PRE3$  with His114, His116, Ser118, Asp120;  $\beta_3/PUP3$  in the case of  $\beta_2/PUP1$  with the residues Asp114, Asp120 and Cis118 and  $\beta_6/C5$  in the case of  $\beta_5/PRE2$  with Ser118, Asp114, Glu120 and Glu122.

Lactacystin is covalently bound to  $\beta_5/PRE2$ . This is in agreement with the observed chemical modification of subunit X of the mammalian proteasome (Fenteany et al., 1995), *Science* 268, 726-730) the homologue of PRE2. Its dimethyl side chain at C10 extends into S1 like a valine or leucine side chain but less deeply than the norleucine side chain of calpain. Lactacystin forms several hydrogen

bridges with atoms of the protein main chain LactN-Gly470, Lact04'-Gly47N, Lact09'-Thr21N, Lact06'-Thr1N. Since these latter interactions can also occur in  $\beta_2$ /PUP1 and  $\beta_1$ /PRE3 which form no covalent complexes with lactacystin, the S1 side group which binds in the hydrophobic S1 pocket of  $\beta_5$ /PRE2 appears to direct the formation of a covalent bond and its stabilization. Thus this side group is an important starting point for the development of inhibitors.

#### 4.5 Specificity

$\beta_5$ /PRE2 has a methionine residue at position 45 which is in contact with the branched side chain of lactacystin in the complex. In the calpain-inhibitor complex the norleucine side chain of calpain pushes the methionine side chain by up to 0.27 nm towards Ile35 which rotates out of the way. This concerted movement makes the S1 pocket more spacious. This is compatible with the observation that lactacystin inhibits the chymotryptic activity towards chromogenic substrates. In a similar manner the chymotryptic activity is reduced in proteasomes with a  $\beta_5$ /PRE2 mutant which cannot be processed from their proform (Chen & Hochstrasser (1996), Cell 86 961-972) and by a mutation in  $\beta_5$ /PRE2 where a substitution of Ala49 by Val in the S1 pocket limits the size (Heinemeyer et al. (1993), J. Biol. Chem. 268, 5115-5120).  $\beta_1$ /PRE3 has an arginine residue in position 45 at the bottom of the S1 pocket which is well suited for glutamate P1 residues. It is most probably the subunit that is associated with the peptidylglutamyl-peptide hydrolysis activity (PGPH) of the proteasome. However, the norleucine side chain also occupies this basic pocket in the calpain inhibitor complex. A high additional density peak was observed which is associated with the

guanidinium side chain and can be interpreted as a chloride or carbonate ion which compensates a non-equalized positive charge.  $\beta_2/\text{PUP1}$  has a glycine as residue 45 and consequently a spacious S1 pocket, the bottom of which is bordered by His35 and Glu53.

We conclude that  $\beta_5/\text{PRE2}$  contains the chymotryptic as well as the tryptic activity whereas  $\beta_1/\text{PRE3}$  contains the PGPH activity but that both pockets are adaptable with regard to size (PRE2) and polarity (PRE3).  $\beta_2/\text{PUP1}$  is suitable for very large P1 residues with a basic character. Mutation analyses have shown that substitutions in  $\beta_4/\text{C11}$  and  $\beta_7/\text{PRE4}$  influence the chymotrypsin-like and the PGPH activity (Heinemeyer et al., (1993) *supra*; Hilt & Wolf (1996), TIBS 21, 96-102, Hilt et al. (1993), J. Biol. Chem. 268, 3479-3486). These subunits are inactive but are located in the vicinity of the subunits  $\beta_5/\text{PRE2}$  and  $\beta_1/\text{PRE3}$  from both rings (figure 4). The substitution of Ser136 by the voluminous Phe in  $\beta_4/\text{C11}$  interferes with the  $\beta$ -trans- $\beta$  contact on helix H3 between  $\beta_4/\text{C11}$  and  $\beta_5/\text{PRE2}$  and can interfere with the neighbouring Thr1 position, as presumably also the deletion of 15 C-terminal residues of  $\beta_7/\text{PRE4}$ , which form extensive contacts with  $\beta_1/\text{PRE3}$  (fig. 3).

#### 4.6 Propeptides and processing

Five  $\beta$  type subunits are synthesized using propeptides of different lengths of up to 75 amino acids which are cleaved during maturation.  $\beta_2/\text{PUP1}$ ,  $\beta_5/\text{PRE2}$  and  $\beta_1/\text{PRE3}$  exhibit an autolysis between Gly-1 - Thr1. This is a process which requires the presence of Thr1, Gly-1 and Lys33. We had already suggested an autolysis within the subunit in which Thr10 $\gamma$  as a nucleophile attacks the

preceding peptide bond (Schmidtke et al. (1996), EMBOJ. 15, 6887-6898).

According to the crystal structure the water NUK is allocated a central role. It is ideally positioned to act as a base for the removal of a proton of Thr<sup>10γ</sup> and to promote nucleophilic addition to the carbonyl carbon of Gly-1. There is no information about the position and orientation of the Gly-1-Thr1 peptide group in the completely processed subunits but we can derive them from partially processed or unprocessed subunits β3/PUP3, β6/C5 and β7/PRE4 which have similar orientations. In these subunits Gly-10 is directed towards the positively charged Lys<sup>33N</sup> and of Gly<sup>47N</sup> which form an oxygen anion hole in analogy to serine proteases in order to distribute the negative charge that is formed when the tetrahedral adduct is formed. A rearrangement to form the ester can take place after proton transfer from the water NUK to Thr<sup>1N</sup> and cleavage of the peptide bond. The nearby residues Ser<sup>1290γ</sup> and Ser<sup>1690γ</sup> support this reaction. Both hydroxyl groups are bound to Asp<sup>166</sup> which is invariant in the active subunits via hydrogen bridges. NUK probably also participates in the ester hydrolysis as an attacking nucleophile which is ultimately incorporated into the product (fig. 5, sections a to c). The Gly-1 residue appears to be essential since a side chain at position -1 would interfere with the protein backbone at position 168 and would force a configuration which is unsuitable for autolysis.

When Thr1 is released the subunits become active. If the catalytic site is not intact as in the subunits β3/PUP3, β6/C5 and β4/C11 in which Thr1 is absent, in β7/PRE4 in which Lys<sup>33</sup> is replaced by Arg and in constructed variants of LMP2, the mammalian homologue of β1/PRE3

(Schmidtke et al. (1996), supra) and of PRE2 (Chen & Hochstrasser (1996), supra) an autolysis at residue 1 does not occur.  $\beta_7/\text{PRE}4$  has both essential residues Gly-1 and Thr1 but in a configuration which differs strongly from that in which the active subunits are found since the Thr1 side chain is pushed away by the larger Arg33 which replaces the lysine residue (figure 4b). The finding of defects in the catalytic activity and in the processing demonstrate the structural lability of the Thr1 site which can be impaired by mutations of neighbouring residues of the same or neighbouring subunits. On the other hand it is also possible that an inactive mutant can become active in the vicinity of active subunits which is in accord with observations that *T. acidophilum* species which have a defect in processing are processed when coexpressed with wild-type protein (Seemüller et al. (1996), supra).

The propeptides play an essential role in the assembly of eukaryotic proteasomes which may be due to direct or indirect effects by participation in interactions between subunits and/or by stabilizing the structure of subunits. The observed structures of the processing intermediates of  $\beta_7/\text{PRE}4(M)$  and  $\beta_6/C5$  and of the unprocessed propeptide  $\beta_3/\text{PUP}3$  indicate that both effects occur since the propeptides are firmly bound to the residue of the protein and interact with other subunits e.g. propeptide  $\beta_7/\text{PRE}4$  with  $\beta_1/\text{PRE}3$  and residues 92 and 115 and propeptide  $\beta_6/C5$  with  $\beta_7/\text{PRE}4$  at 91 and 116.

#### 4.7 Entry into and exit from the proteasome particle

The hydrolytic activity of the proteasome is associated with Thr1 and the  $\beta$  ring surfaces in the interior of the

$\beta$  cavity that defines the hydrolytic chamber. The substrate must penetrate into the particle and the product must be released. In the case of the proteasome from *T.acidophilum* two entry openings with a diameter of about 1.3 nm are open at the ends of the cylindrical particles which are bordered by a ring surface of bend-forming segments Tyr126-Gly-Gly-Val of seven identical  $\alpha$  subunits. The N-terminal residues 1 to 12 are disordered in this protein.

In contrast the hydrolytic chamber of the 20S proteasome of yeast is almost inaccessible. The N-termini of the subunits  $\alpha$ 1/C7,  $\alpha$ 2/Y7,  $\alpha$ 3/Y13,  $\alpha$ 6/PRE5 and  $\alpha$ 7/C1 protrude into the opening and fill it completely with several layers of closely interwoven sidechains (figure 8). Hence there is no access to the interior of the particle from the cylinder ends without a considerable rearrangement. There some narrow side windows in particular at the interface between the  $\alpha$  and  $\beta$  rings which are more permeable than in the *T.acidophilum* proteasome since smaller side chains are present there. These openings are mainly located between the tooth-like helix H1-bend-helix H2 motifs of the  $\alpha$ - $\beta$  interface (see figure 4a by Löwe et al. (1995), supra) and lead to the N-terminal threonine residues of the active centre. They are covered by polar and charged amino acid side chains which can move in order to generate openings of about 1 nm diameter and possibly allow the passage of unfolded stretched polypeptide chains. The 19S particle which is responsible for the ATP dependency and ubiquitin dependency of proteolysis by the proteasome is attached to the particles to form the 26S proteasome. The association leads to a strong activation of peptide hydrolysis (Hoffman and Rechsteiner (1994), J. Biol. Chem. 269, 1690-1695). The proteasome regulator PA28 is

bound in a similar manner to  $\alpha$  type subunits (Kania et al. (1996), Euro. J. Biochem. 236, 510-516). It accelerates peptide cleavage and improves the antigen processing. Both regulatory factors could open the entry openings in a controlled manner *in vivo*.

#### 4.8 Generation of MHC class I peptides

The 20S proteasome generates peptide products with a narrow length distribution, mainly octapeptide or nonapeptides, a size range which is optimal for binding MHC class I molecules (York & Rock (1996), Annu. Rev. Immunol. 14, 369-396). In vitro experiments have shown that peptides generated from intact proteins by 20S proteasomes are presented by MHC class I molecules (Dick et al. (1994) Immunol. 152, 3884-3894; Niedermann et al. (1996), Proc. Natl. Acad. Sci. USA 93, 8572-8577). In an *in vivo* experiment it was shown that proteasome inhibitors inhibit the MHC class I presentation of protein antigens (Rock et al. (1994), Cell 78, 761-771) and that the number of the MHC class I molecules present on the cell surface is regulated by the inducible proteasome subunits  $\beta$ 5i/LMP7 and  $\beta$ 1i/LMP2 as shown in mice with site-directed deletions of the genes coding for these proteins (Fehling et al. (1994), Science 265, 1234-1237). LMP2 and LMP7 replace the constitutively expressed subunits after IFN- $\gamma$  stimulation.

MHC class I peptides usually have basic or hydrophobic C-terminal residues (see the review article by Engelhard (1994), Curr. Opin. Immunol. 6, 13-23). The LMP2/7 substitution presumably changes the distribution of peptides so that a larger proportion of the peptides preferred by MHC class I molecules is generated. LMP2

replaces Y the human homologue of  $\beta 1/\text{PRE}3$ , LMP7 replaces X the homologue of  $\beta 5/\text{PRE}2$ . All members of this subfamily exhibit a high degree of sequence identity but  $\beta 1i/\text{LMP}2$  has two conspicuous differences compared to  $\beta 1/\text{PRE}3$  in the S1 pocket:  $\text{Thr}31 \rightarrow \text{Phe}$  and  $\text{Arg}45 \rightarrow \text{Leu}$ . The substitution of Arg by Leu makes the pocket unpolar and the substitution of Thr by Phe makes it narrower so that the PGPH activity should be reduced and the chymotryptic activity should be increased if  $\beta 1i/\text{LMP}2$  replaces the mammalian homologue for  $\beta 1/\text{PRE}3$ . This is actually observed (Gaczynska et al. (1993), Nature 365, 264-267; Driscoll et al. (1993), Nature 365, 262-264) when LMPs are induced by treatment with IFN- $\gamma$ . The opposite effect is found in cell lines which lack the LMP2 and LMP7 genes and in mutant mice with a disruption of the LMP2 gene (Van Kaer et al. (1994), Immunity 1, 533-541). Replacement of the mammalian homologues of  $\beta 5/\text{PRE}2$  and  $\beta 2/\text{PUP}1$  by LMP7 and MECL1 does not directly influence the S1 pockets and their effect cannot be due to the change in the specificity in P1 as is found for LMP2.

In the yeast 20S proteasome the subunits  $\beta 7/\text{PRE}4$  and  $\beta 6/\text{C}5$  are partially processed at residues -8 and -9. As a result octapeptide or nonapeptide products are formed which are not released from the enzyme. Both peptides have similar conformations with a thickening which subdivides two sections with extended conformations which is similar to the conformation of MHC class I-bound peptides. The similarity is quantified with rms deviations for all atoms of 0.23 nm and for the  $C^\alpha$  atoms of 0.13 nm by comparing the propeptide of  $\beta 6/\text{C}5$  with a viral peptide nonamer in a complex with its MHC class I receptor (Madden et al. (1992), Nature 321-325) and

allows the conclusion that preferred local conformations play a role in the generation (by the proteasome) and presentation (by MHC class I molecules) of immune-dominant peptide epitopes.

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attachment 1

New Claims

1. Process for isolating a purified eukaryotic proteasome preparation comprising the steps:
- (a) production of a crude extract by lysing eukaryotic cells,
  - (b) separation of insoluble components from the crude extract,
  - (c) chromatographic separation into fractions by means of an ion exchange medium,
  - (d) testing the fractions obtained in step (c) and collecting the active fractions,
  - (e) chromatographic separation over hydroxyapatite,
  - (f) testing the fractions obtained in step (e) and collecting the active fractions,
  - (g) concentrating the pooled fractions,
  - (h) chromatographic separation over a gel filtration medium and
  - (i) testing the fractions obtained in step (h) and collecting the active fractions,
- wherein each testing of the fractions in steps (d), (f) or/and (i) comprises two determinations of the proteolytic activity one of which is carried out in the absence and the other in the presence of a proteasome inhibitor.

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2. Process as claimed in claim 1,  
**wherein**  
yeast cells are used.
3. Process as claimed in claim 2,  
**wherein**  
lactacystin is used as the proteasome inhibitor.
4. Process as claimed in one of the claims 1 to 3,  
**wherein**  
at least one of the chromatographic separation  
steps is carried out in a FPLC system.
5. Process as claimed in one of the claims 1 to 4,  
also comprising the crystallization of the purified  
proteasome preparation.
6. Purified eukaryotic proteasome preparation  
obtainable by the process as claimed in one of the  
claims 1 to 4.
7. Purified eukaryotic proteasome preparation as  
claimed in claim 6 in a crystallizable form.
8. Purified crystallized eukaryotic proteasome  
preparation,  
**wherein**  
it allows a crystallographic analysis at a  
resolution of 0.28 nm or higher.

9. Purified crystallized eukaryotic proteasome preparation as claimed in claim 8,  
**wherein**  
it allows a crystallographic analysis at a resolution of 0.24 nm.
10. Preparation as claimed in claim 8 or 9,  
**wherein**  
the crystal contains a proteasome inhibitor.
11. Preparation as claimed in claim 10,  
**wherein**  
the inhibitor is a tripeptide aldehyde or lactacystin.
12. Preparation as claimed in one of the claims 6 to 11,  
**wherein**  
it contains a proteasome from a yeast.
13. Preparation as claimed in claim 12,  
**wherein**  
it contains a proteasome from *Saccharomyces cerevisiae*.
14. Preparation as claimed in one of the claims 6 to 13,  
**wherein**  
it contains a complex of 28 subunits which contains two molecules each of 7 different  $\alpha$  type subunits and 7 different  $\beta$  type subunits.

15. Use of the purified eukaryotic proteasome preparation as claimed in one of the claims 6 to 14 to identify and isolate new proteasome inhibitors.
16. Use of data from the crystal structure of crystallized eukaryotic proteasome preparations as claimed in one of the claims 8 to 14 to identify and isolate new proteasome inhibitors.
17. Use of crystal structural data from the region of the proteasome pockets S1 of the subunits  $\beta_1/\text{PRE}3$ ,  $\beta_2/\text{PUP}1$  or/and  $\beta_5/\text{PRE}2$  to identify and isolate new proteasome inhibitors.
18. Use as claimed in one of the claims 15 to 17 in a computer-aided modelling programme.
19. Use as claimed in claim 18, comprising a step of homology modelling in which the crystal structural data of a yeast proteasome are modified with amino acid sequences from the human proteasome.
20. Process for providing new proteasome inhibitors, **wherein**  
compounds are identified based on data from the crystal structure of crystallized eukaryotic proteasome preparations as claimed in one of the claims 8 to 14 which have a three-dimensional structure which is complementary to the proteasome pocket S1 of the subunits  $\beta_1/\text{PRE}3$ ,  $\beta_2/\text{PUP}1$  or/and  $\beta_5/\text{PRE}2$ .

### **Abstract**

The invention concerns a process for isolating a purified eukaryotic crystallizable proteasome preparation and the proteasome preparation obtainable by the process. The invention in addition concerns a purified eukaryotic proteasome preparation in a crystallized form. The crystal data from this proteasome preparation can be used to identify and isolate new proteasome inhibitors especially with the aid of computer-aided modelling programs.

Figure 1a

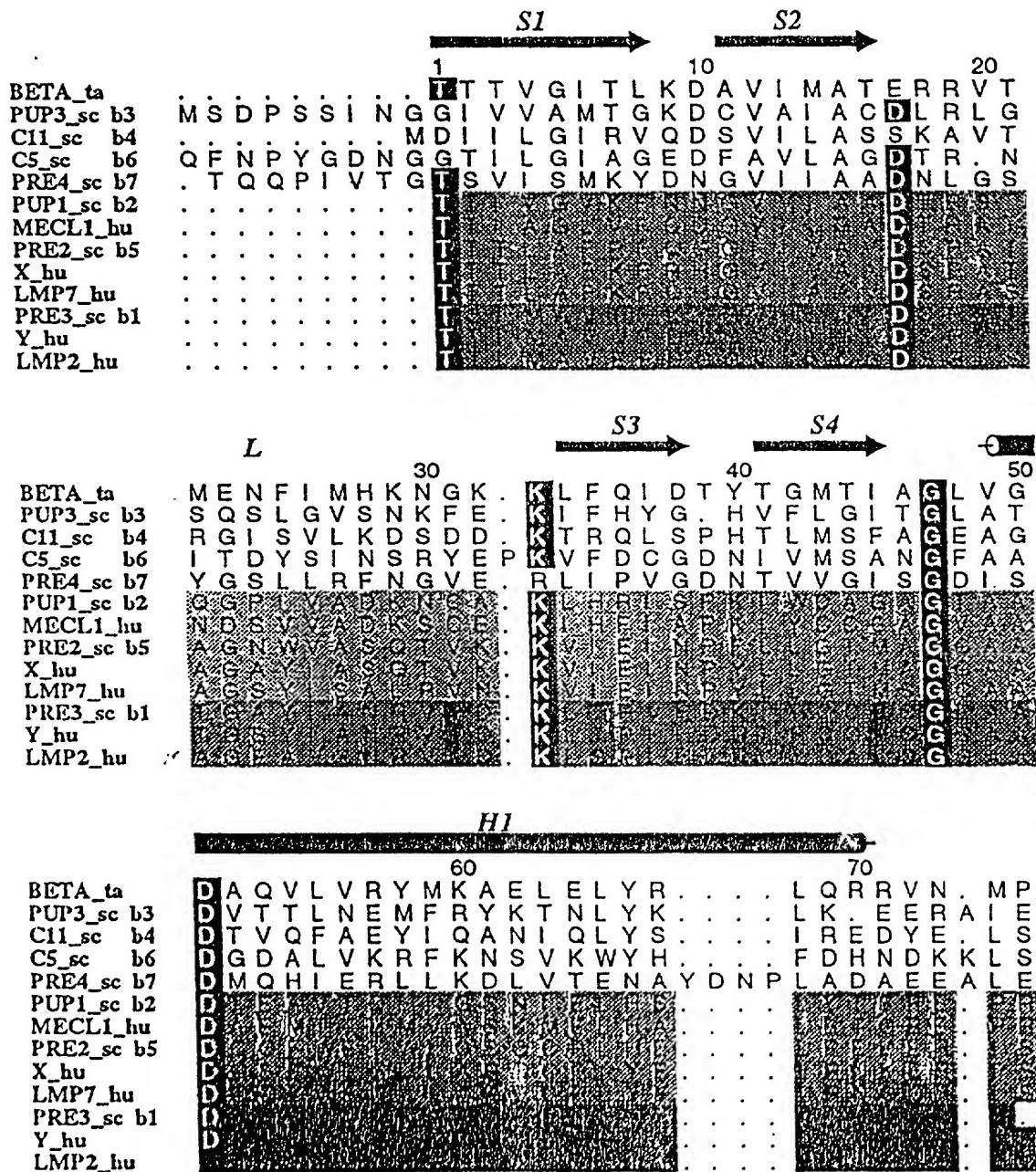
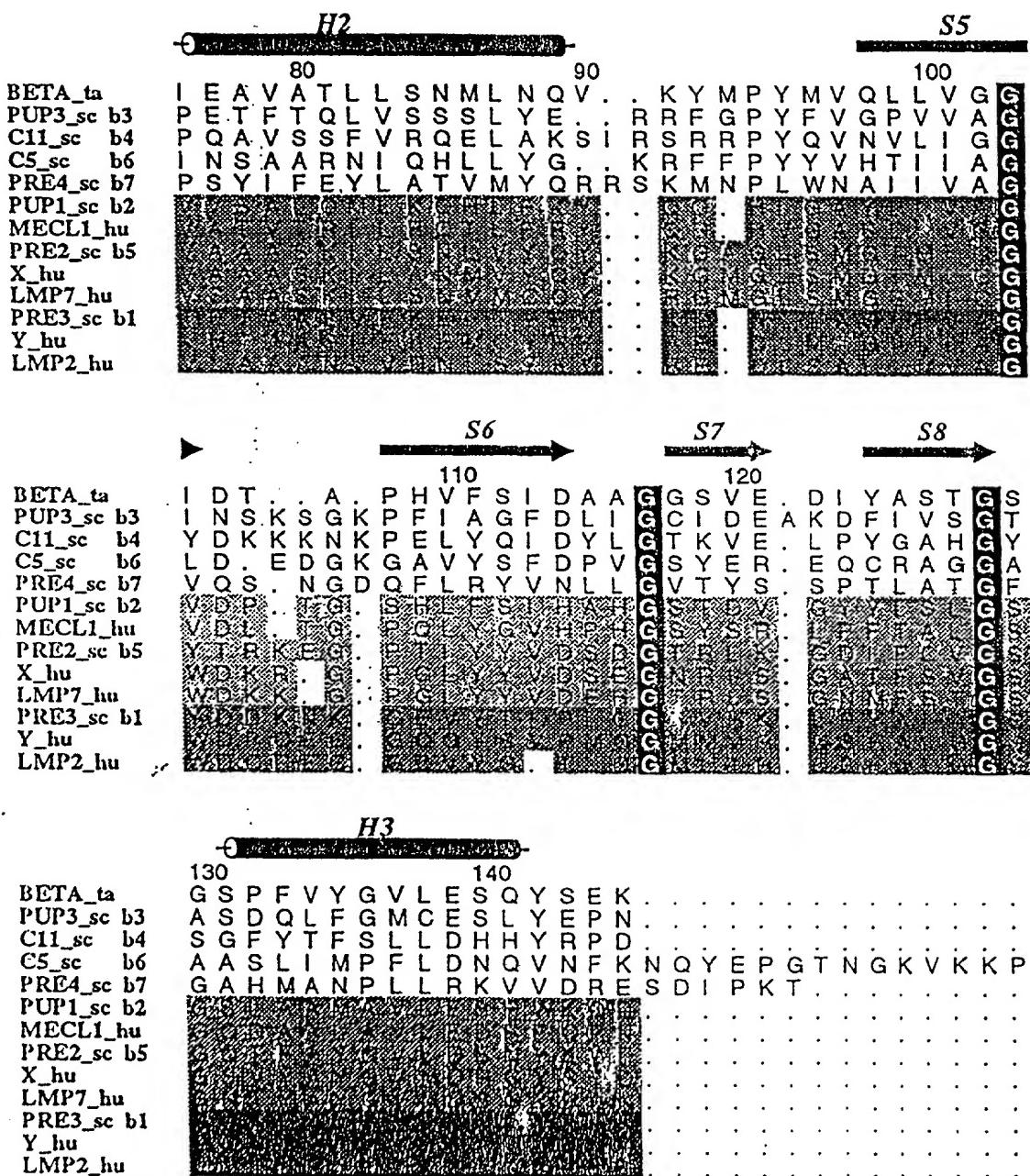


Figure 1b



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Figure 1c

Sequence alignment of H4, S9, S10, and H5 domains.

	<i>H4</i>				
BETA_ta	...	MTVDEGV	DLVIRAI	SAAKQRD	SASGGM
PUP3_sc b3	...	LEPEDLFET	ISQALLNAA	ADRDA	LSGWG
C11_sc b4	...	TTTEEGLDLL	KLCVQELEKRM	MPMDFKG	
C5_sc b6	LKYLSVEEV	IKLVRDSFTSATE	RHIQVG	DGDG	
PRE4_sc b7	TVQVAEEAI	VNAMRVLYYR	DARSSRN		
PUP1_sc b2					
MECL1_hu					
PRE2_sc b5					
X_hu					
LMP7_hu					
PRE3_sc b1					
Y_hu					
LMP2_hu					
150 160 170					
180 190 200					
	<i>S9</i> → <i>S10</i> → <i>H5</i>				
BETA_ta	I D V A V I T R K D G Y V Q L P T D Q I E S R I R K L G L I				
PUP3_sc b3	A V V Y I I K K D E V V K R Y L K M R Q D . . . . .				
C11_sc b4	V I V K I V D K D G I R Q V D D F Q A Q . . . . .				
C5_sc b6	L E I L I V T K D G V R K E F Y E L K R D . . . . .				
PRE4_sc b7	F S L A I I D K N T G L T F K K N L Q V E N M K W D F A K D				
PUP1_sc b2	Y D V C V D M E G K D A E Y T E N Y L P D N V R E E K C G S				
MECL1_hu	M D A C V I T K T I G A K L P T T S S P I E P V K R E G D				
PRE2_sc b5	N N Y H V I T E D G W I P H G N T A G E F F W V V E P E				
X_hu	N V I H I : R E D G V I P K S S N T A P E E K V V S C D				
LMP7_hu	N V I H M : K E R G V S I P E S T I L S D E E D E P E				
PRE3_sc b1					
Y_hu					
LMP2_hu					
210 220					
BETA_ta	L .				
PUP3_sc b3	. .				
C11_sc b4	. .				
C5_sc b6	. .				
PRE4_sc b7	I K G Y G T Q K I .				
PUP1_sc b2	Y D V C V D M E G K D A E Y T E N Y L P D N V R E E K C G S				
MECL1_hu	M D A C V I T K T I G A K L P T T S S P I E P V K R E G D				
PRE2_sc b5	N N Y H V I T E D G W I P H G N T A G E F F W V V E P E				
X_hu	N V I H I : R E D G V I P K S S N T A P E E K V V S C D				
PRE3_sc b1					
Y_hu					
LMP2_hu					

Figure 1d

BETA\_ta  
PUP3\_sc b3  
C11\_sc b4  
CS\_sc b6  
PRE4\_sc b7  
PUP1\_sc b2  
MECL1\_hu  
PRE2\_sc b5  
X\_hu  
LMP7\_hu  
PRE3\_sc b1  
Y\_hu  
LMP2\_hu

Figure 2

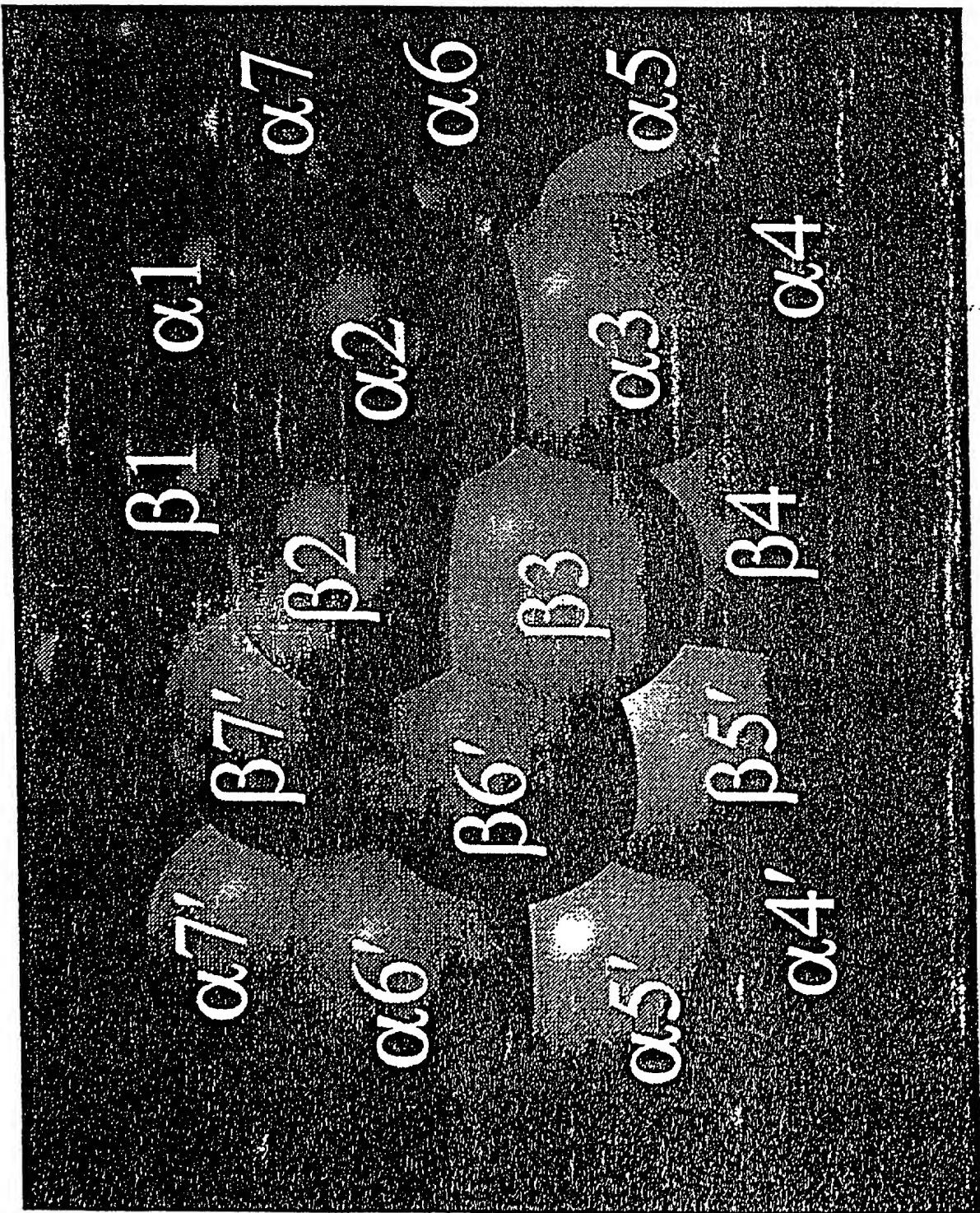
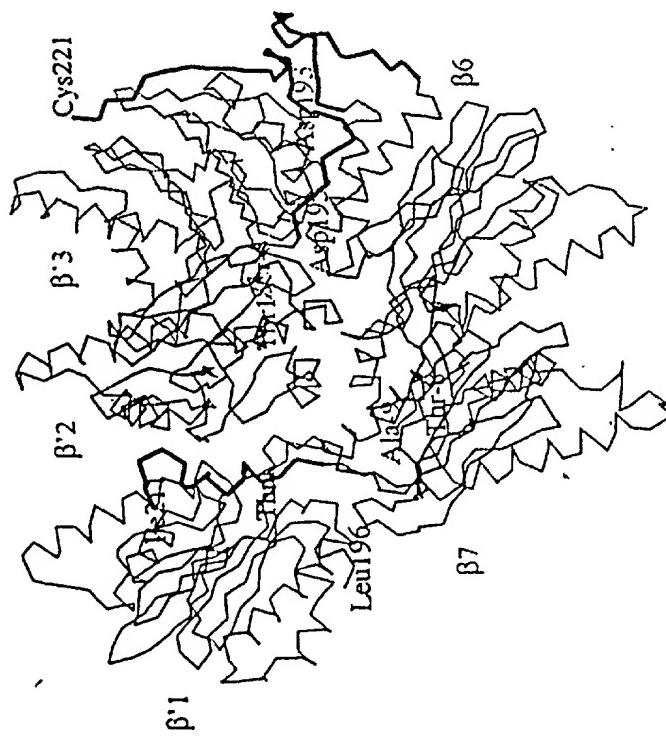
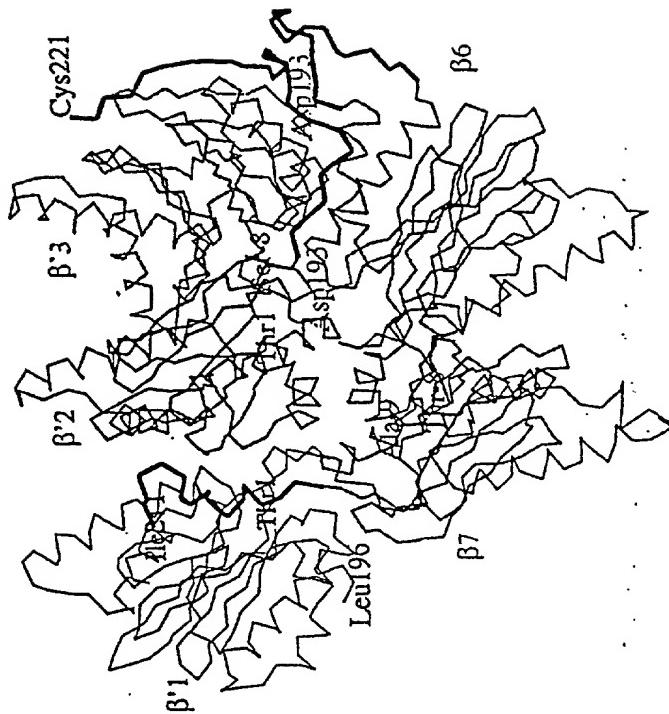


Figure 3



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Figure 4a

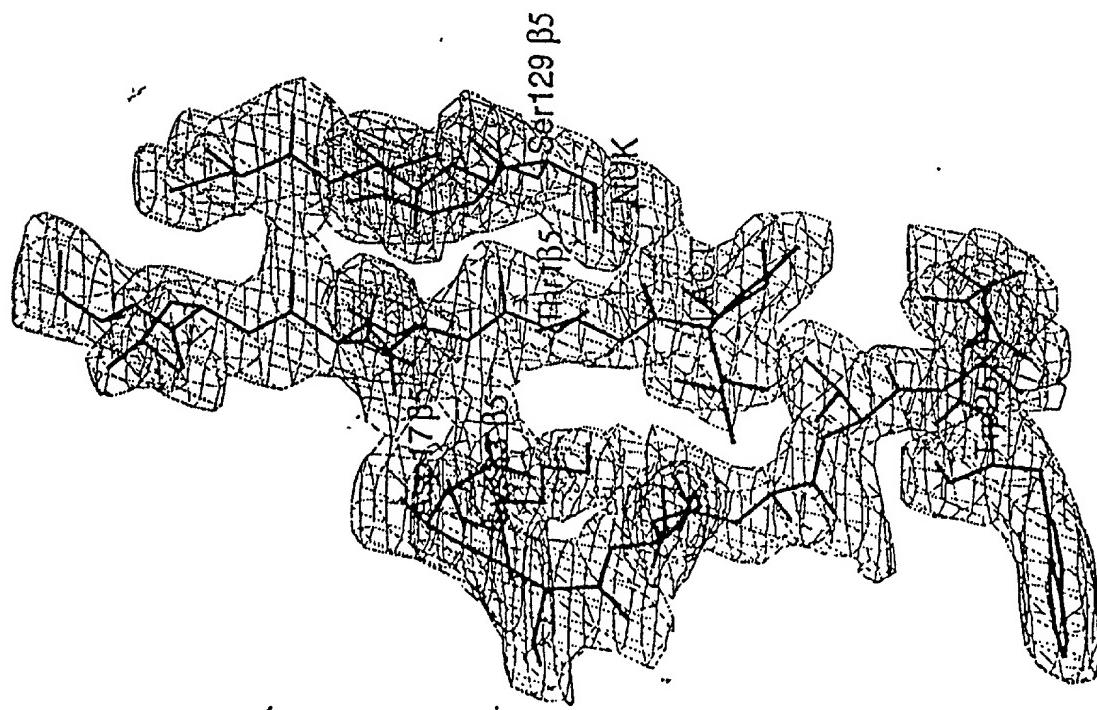
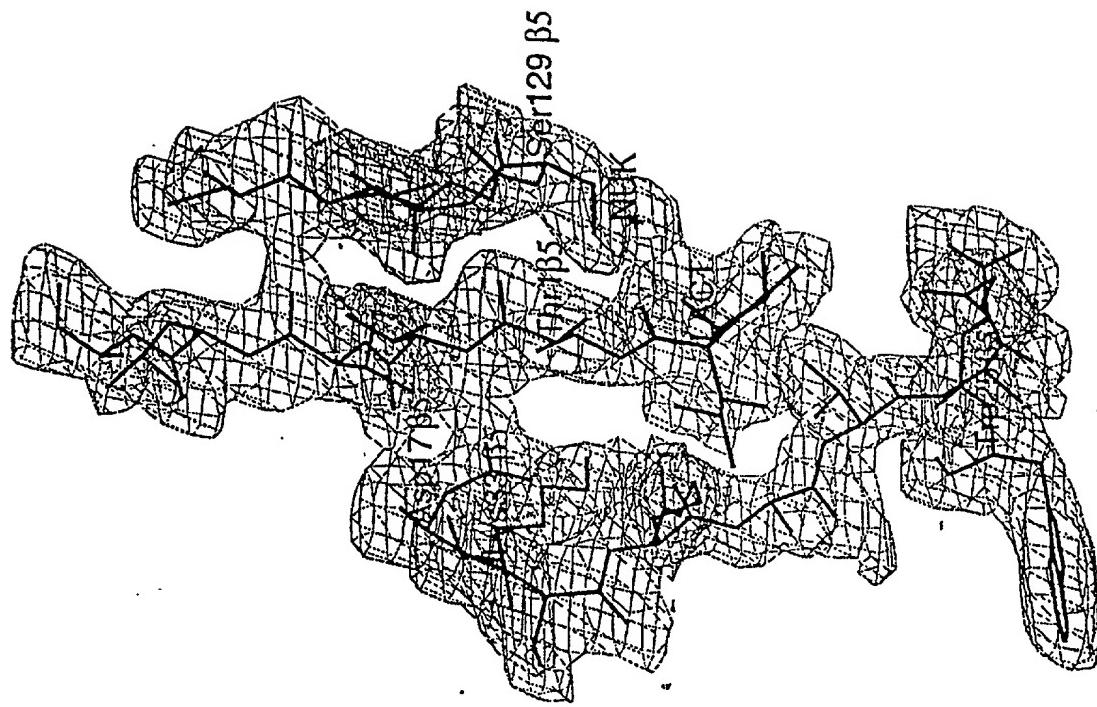


Figure 4b

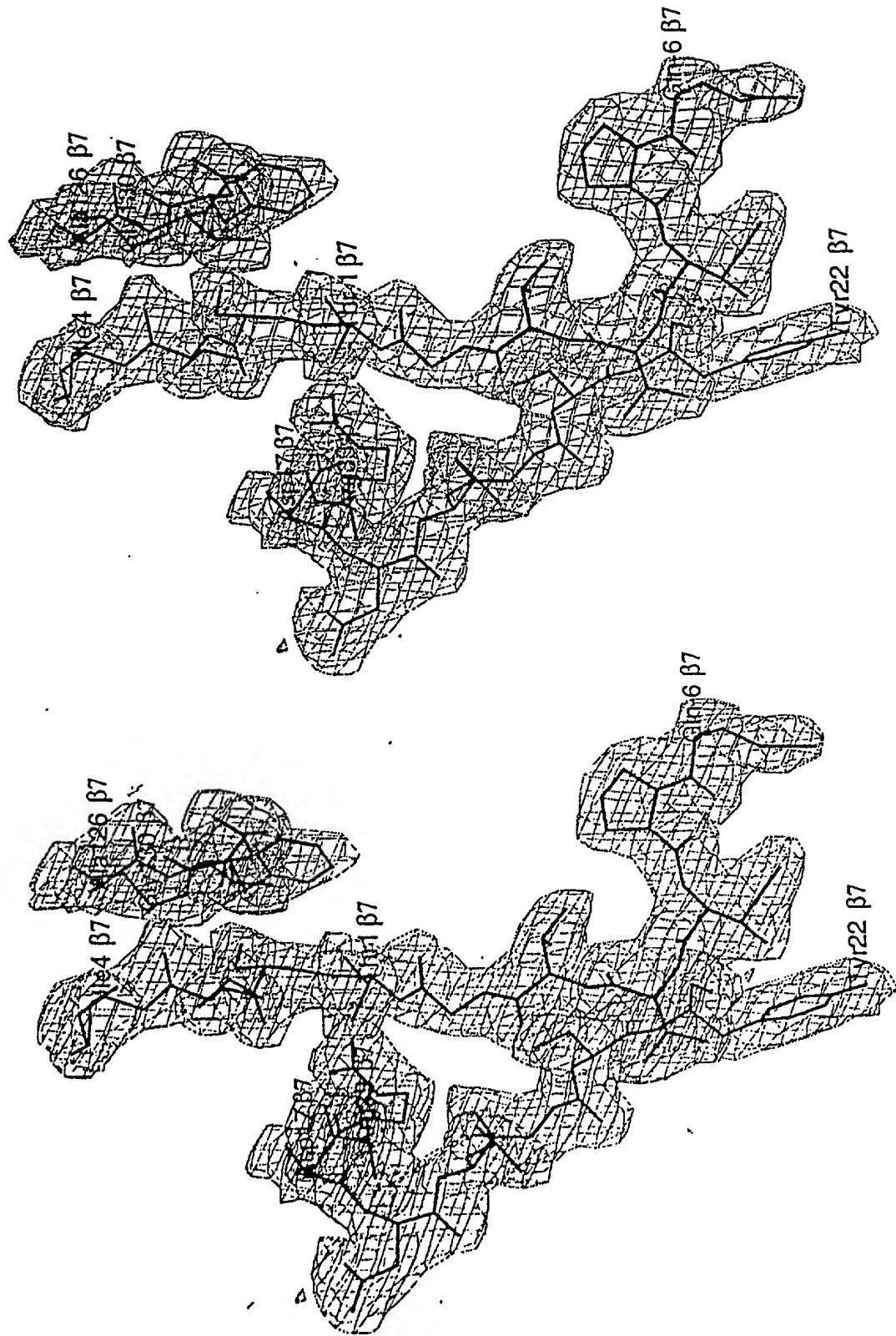


Figure 5

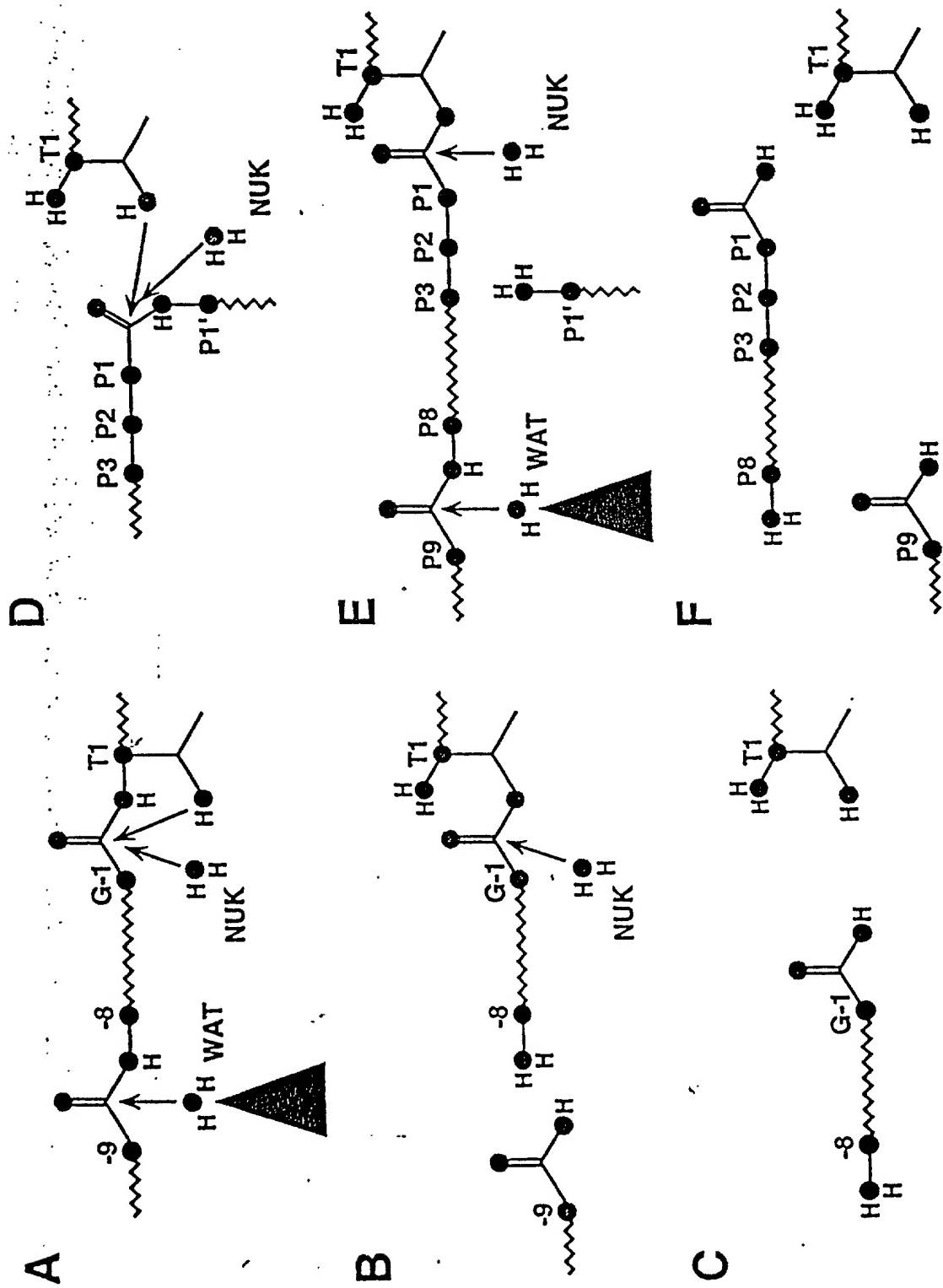


Figure 6a

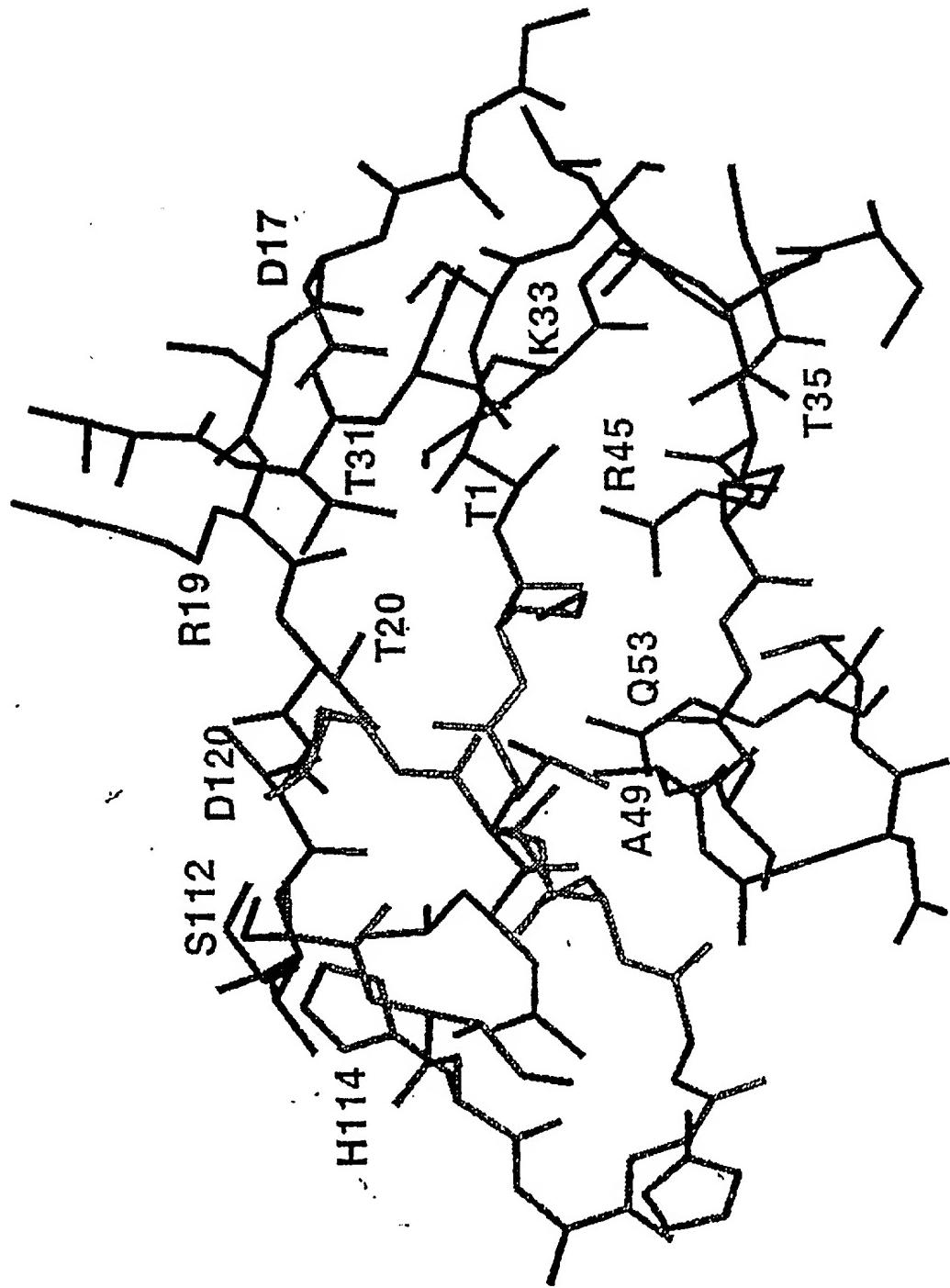
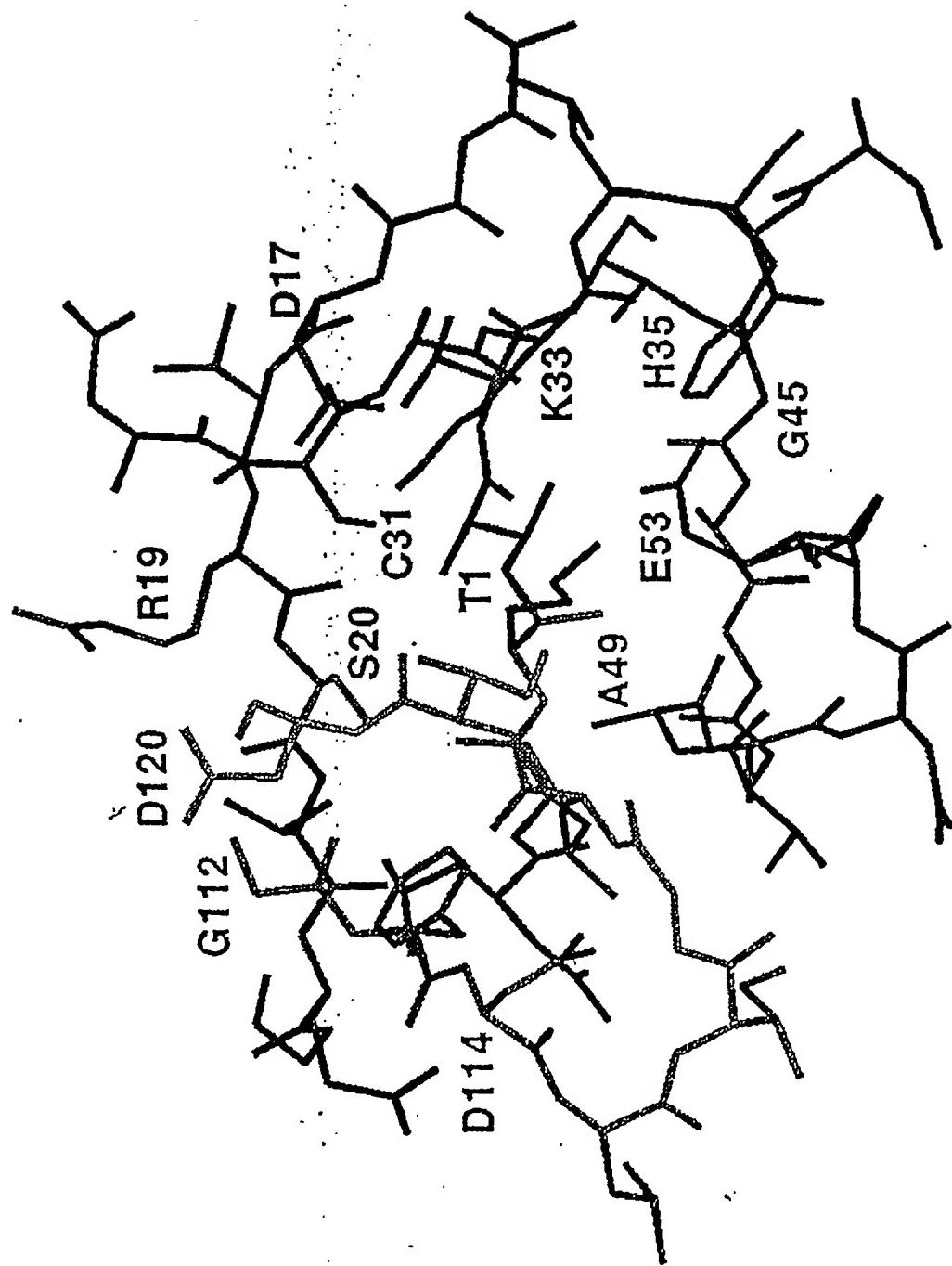


Figure 6b



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Figure 6c

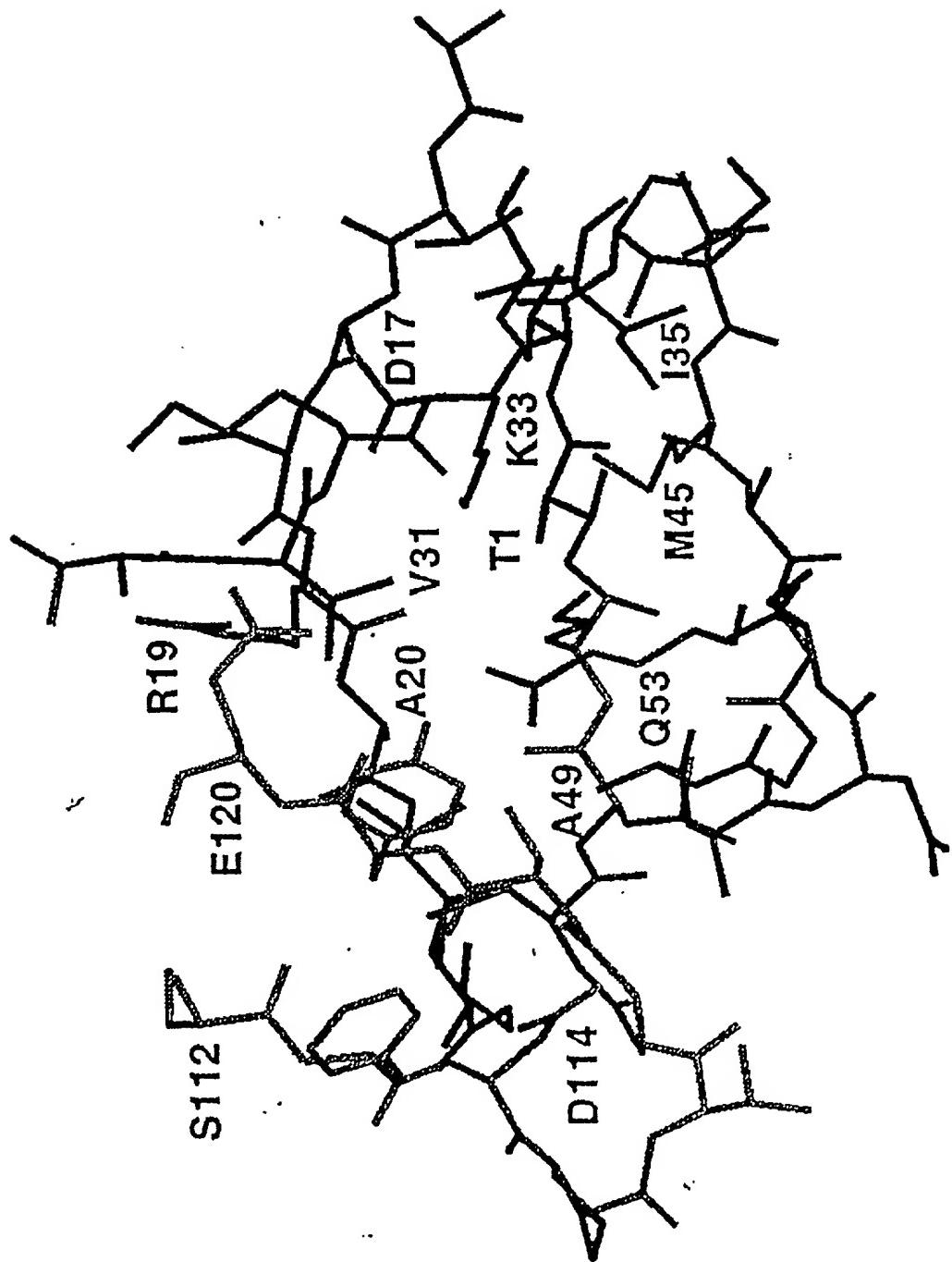
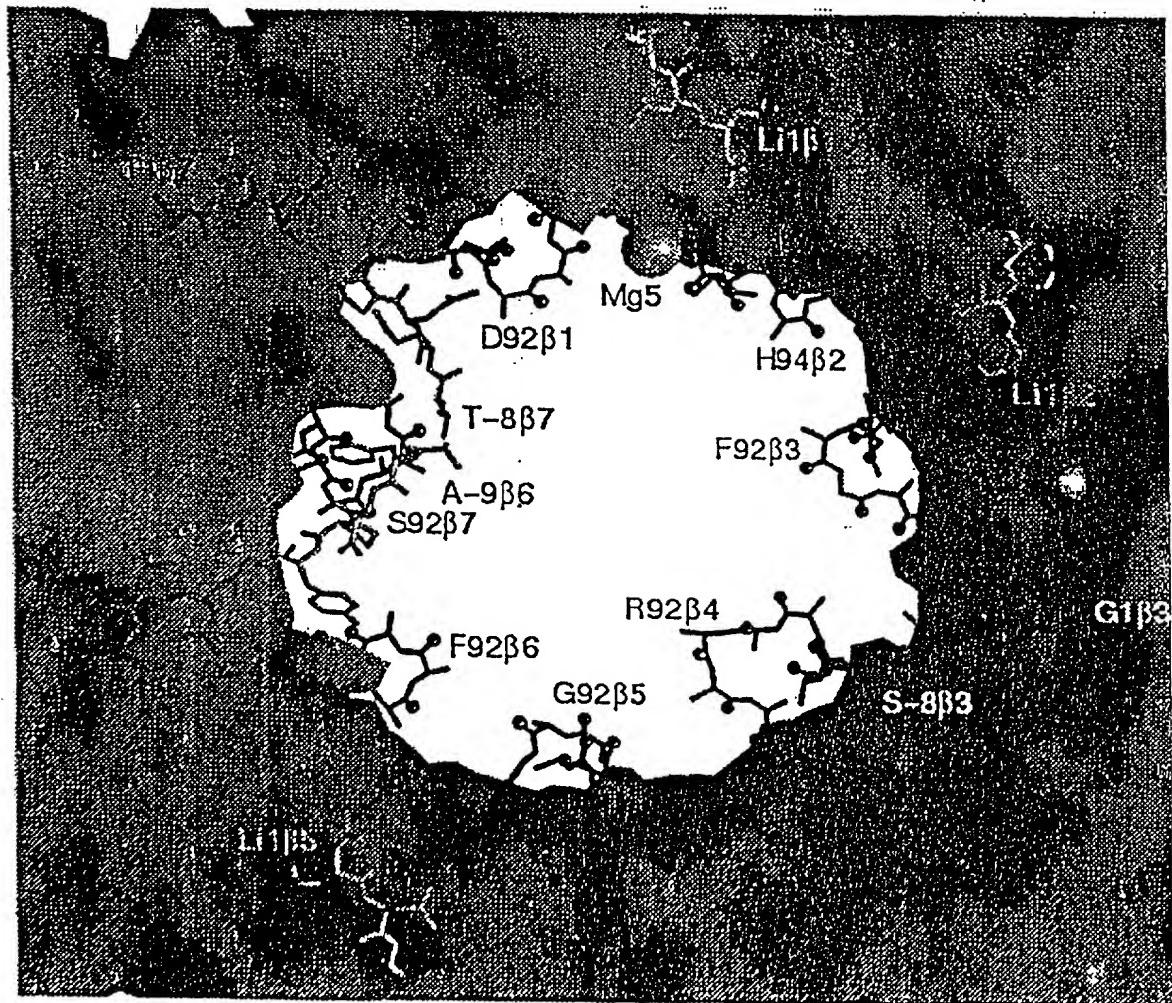


Figure 7

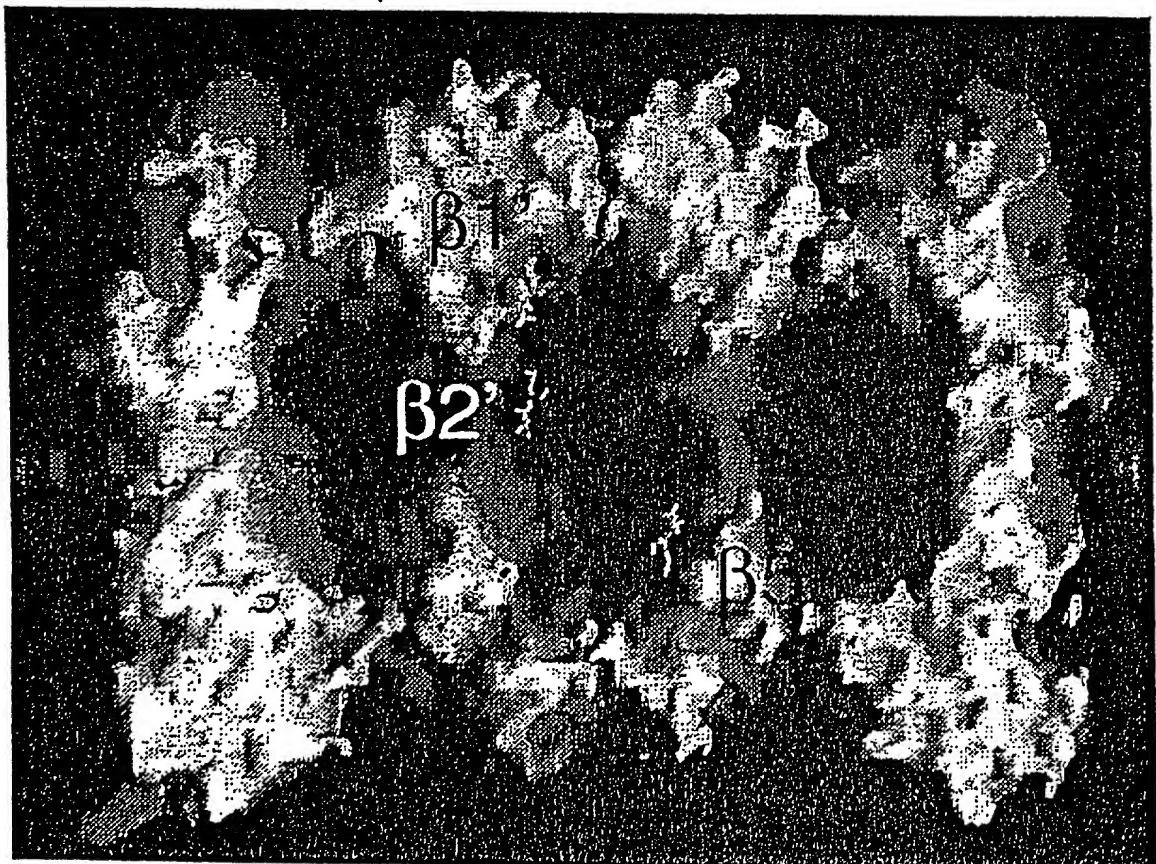


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Figure 8



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# Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled  
(Insert Title) Process for the purification and crystallization of proteasome

the specification of which

(Check one  
of blocks  
1, 2 or 3.  
See note A  
on back of  
this page)

1.  is attached hereto.
2.  was filed on March 20, 1998 as  
International PCT Application Serial No. PCT/EP98/01653  
and was amended on Feb. 11, 1999  
(if applicable)
3.  was filed on \_\_\_\_\_ as  
U.S. Application Serial No. \_\_\_\_\_  
and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application for which priority is claimed:

	97 104 877.2 (Number)	EP (DE) (Country)	21 March 1997 (Day/Month/Year Filed)	Priority Claimed <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
(List prior foreign applications. See note B on back of this page)	_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
	_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
	_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No

(See Note C on back  
of this page)  See attached list for additional prior foreign applications

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT International filing date of this application:

(List prior U.S. Applications or PCT International applications designating the U.S.)	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

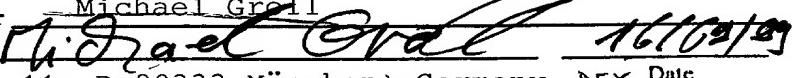
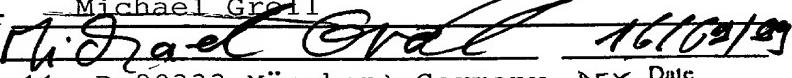
And I hereby appoint as principal attorneys David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980; Martin S. Postman, Reg. No. 18,570; E. Marcie Emas, Reg. No. 32,131; Michael G. Gilman, Reg. No. 19,114; Douglas H. Goldhush, Reg. No. 33,125; Kevin C. Brown, Reg. No. 32,402; Monica Chin Kitts, Reg. No. 36,105; Sharon N. Klesner, Reg. No. 36,335, and John R. Fuisz, Reg. No. 37,327.

Please direct all communications to the following address: NIKAIKO, MARMELSTEIN, MURRAY & ORAM

Metropolitan Square  
655 Fifteenth Street, N.W., Suite 330 - G Street Lobby  
Washington, D.C. 20005-5701  
(202) 638-5000 Fax: (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note D  
on back of  
this page)

100 Full name of sole or first inventor Michael Grell   
 Inventor's signature Michael Grell   
 Residence Türkenstr. 11, D-80333 München, Germany  Date  
 Citizenship German  
 Post Office Address same as above

Full name of second joint inventor, if any Robert Huber

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence Schlesierstr. 11, D-82110 Germering, GermanyCitizenship GermanPost Office Address same as above

3-00

Full name of third joint inventor, if any Lars DitzelInventor's signature (Lars Ditzel) Date 21.10.1999Residence Asternstr. 12, D-80689 München, GermanyCitizenship GermanPost Office Address same as above

000

Full name of fourth joint inventor, if any Richard Engh

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence Herbststr. 4, D-82234 Wessling, GermanyCitizenship American (USA)Post Office Address same as above

000

Full name of fifth joint inventor, if any \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_

Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

000

Full name of sixth joint inventor, if any \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_

Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

000

Full name of seventh joint inventor, if any \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_

Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

000

Full name of eighth joint inventor, if any \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_

Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

2-00

Full name of second joint inventor, if any Robert Huber Inventor's signature R. Huber Date 16.9.99  
Residence Schlesierstr. 13, D-82110 Germerring, Germany REX  
Citizenship German  
Post Office Address same as above

Full name of third joint inventor, if any Lars Ditzel

Inventor's signature \_\_\_\_\_  
Residence Asternstr. 12, D-80689 München, Germany Date  
Citizenship German  
Post Office Address same as above

4-00

Full name of fourth joint inventor, if any Richard Engh Inventor's signature Richard A. Engh Date 16. Sept 99  
Residence Herbststr. 4, D-82234 Wessling, Germany REX  
Citizenship American (U.S.A.)  
Post Office Address same as above

Full name of fifth joint inventor, if any \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date  
Residence \_\_\_\_\_  
Citizenship \_\_\_\_\_  
Post Office Address \_\_\_\_\_

Full name of sixth joint inventor, if any \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date  
Residence \_\_\_\_\_  
Citizenship \_\_\_\_\_  
Post Office Address \_\_\_\_\_

Full name of seventh joint inventor, if any \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date  
Residence \_\_\_\_\_  
Citizenship \_\_\_\_\_  
Post Office Address \_\_\_\_\_

Full name of eighth joint inventor, if any \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date  
Residence \_\_\_\_\_  
Citizenship \_\_\_\_\_  
Post Office Address \_\_\_\_\_